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<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>(21) International Application Number: PCT/US98/27805</p> <p>(22) International Filing Date: 29 December 1998 (29.12.98)</p> <p>(30) Priority Data:</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">09/000,676</td> <td style="width: 30%;">30 December 1997 (30.12.97)</td> <td style="width: 40%;">US</td> </tr> <tr> <td>09/137,430</td> <td>21 August 1998 (21.08.98)</td> <td>US</td> </tr> </table> <p>(71) Applicant: ENZON, INC. [US/US]; 20 Kingsbridge Road, Piscataway, NJ 08854 (US).</p> <p>(72) Inventors: GREENWALD, Richard, B.; 113 Hickory Road, Somerset, NJ 08873 (US). CHOE, Yun, E.; 2 Gemma Court, Piscataway, NJ 08854 (US). PENDRI, Annapurna; 108 Winston Drive, Matawan, NJ 07747 (US).</p> <p>(74) Agents: MERCANTI, Michael, N. et al.; Roberts & Mercanti, L.L.P., P.O. Box 484, Princeton, NJ 08542-0484 (US).</p> </div> <div style="width: 48%;"> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p> </div> </div>			09/000,676	30 December 1997 (30.12.97)	US	09/137,430	21 August 1998 (21.08.98)	US
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<p>(54) Title: TRIALKYL-LOCK-FACILITATED POLYMERIC PRODRUGS OF AMINO-CONTAINING BIOACTIVE AGENTS</p> <div style="text-align: center; margin: 20px 0;"> <p style="margin-top: 10px;">(I)</p> </div>								
<p>(57) Abstract</p> <p>The present invention is directed to double prodrugs containing polymeric-based transport forms. These polymeric compounds comprise the formula (I), wherein: B is H, OH, OSiR₁₃, a residue of an amine-containing target moiety or a residue of a hydroxyl-containing moiety; L₁ and L₂ are bifunctional linking moieties; Y₂ is O or S; R₂ is selected from the group consisting of C₁₋₆ alkyls, C₃₋₁₂ branched alkyls, C₃₋₈ cycloalkyls, C₁₋₆ substituted alkyls, C₃₋₈ substituted cycloalkyls, aryls, substituted aryls, aralkyls, C₁₋₆ heteroalkyls, substituted C₁₋₆ heteroalkyls, C₁₋₆ alkoxy, phenoxy, and C₁₋₆ heteroalkoxy; R₉, R₁₀, R₁₃ are independently one of hydrogen, C₁₋₆ alkyls, C₃₋₁₂ branched alkyls, C₃₋₈ cycloalkyls, C₁₋₆ substituted alkyls, C₃₋₈ substituted cycloalkyls, aryls, substituted aryls, aralkyls, C₁₋₆ heteroalkyls, substituted C₁₋₆ heteroalkyls, C₁₋₆ alkoxy, phenoxy, and C₁₋₆ heteroalkoxy; Ar is a moiety which when included in formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group; (m) is zero or one; and R₁₁ is a polymer residue. The first prodrug is generated when the polymeric portion of the double prodrug is cleaved and the parent molecule is generated rapidly thereafter <u>in vivo</u>, as a result of a trialkyl lock type lactonization-type reaction.</p>								

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TRIALKYL-LOCK-FACILITATED POLYMERIC PRODRUGS OF AMINO-CONTAINING BIOACTIVE AGENTS

TECHNICAL FIELD

5 The present invention relates to double prodrugs. In particular, the invention relates to polymeric-based double prodrugs having reversible linkages involving amino or hydroxyl moieties of chemical compounds and biologically active materials such as enzymes, proteins and the like.

BACKGROUND OF THE INVENTION

10 Over the years, several methods of administering biologically-effective materials to mammals have been proposed. Many medicinal agents are available as water-soluble salts and can be included in pharmaceutical formulations relatively easily. Problems arise when the desired medicinal agent is either insoluble in aqueous fluids or is rapidly degraded in vivo. For example, alkaloids are often especially difficult to solubilize.

15 One way to solubilize medicinal agents is to include them as part of a soluble prodrug. Prodrugs include chemical derivatives of a biologically-active parent compound which, upon administration, eventually liberate the parent compound in vivo. Prodrugs allow the artisan to modify the onset and/or duration of action of an agent in vivo and can modify the transportation, distribution or solubility of a drug in the body. Furthermore, prodrug formulations often reduce the toxicity and/or
20 otherwise overcome difficulties encountered when administering pharmaceutical preparations. Typical examples of prodrugs include organic phosphates or esters of alcohols or thioalcohols. See Remington's Pharmaceutical Sciences, 16th Ed., A. Osol, Ed. (1980), the disclosure of which is incorporated by reference herein.

25 Prodrugs are often biologically inert, or substantially inactive, forms of the parent or active compound. The rate of release of the active drug, i.e. the rate of

hydrolysis, is influenced by several factors but especially by the type of bond joining the parent drug to the modifier. Care must be taken to avoid preparing prodrugs which are eliminated through the kidney or reticular endothelial system, etc. before a sufficient amount of hydrolysis of the parent compound occurs. By incorporating a polymer as part of the prodrug system, one can increase the circulating half-life of the drug.

Although the above-mentioned concept of prodrug-based delivery systems has proven to be useful in many instances, there are nonetheless situations where alternatives are desired. For example, Bundgaard in "The Double Prodrug Concept and Its Applications" in Advanced Drug Delivery Reviews, 3 (1989) 39-65, (the contents of which are hereby incorporated by reference) pointed out that in many cases it is difficult to obtain a prodrug which has the proper combination of adequate stability in vitro and high susceptibility to regenerate the parent drug in vivo. As pointed out by Bundgaard, a promising means of overcoming some of the previously encountered shortcomings involves the use of cascade latentiation or "pro-prodrugs". In such systems, the hydrolytic reaction sequence involves a first step which usually is an enzymatic cleavage and the second involves a non-enzymatic hydrolysis that occurs only after the first has taken place. The use of polymeric-based transport systems as part of cascade latentiation technology was not disclosed.

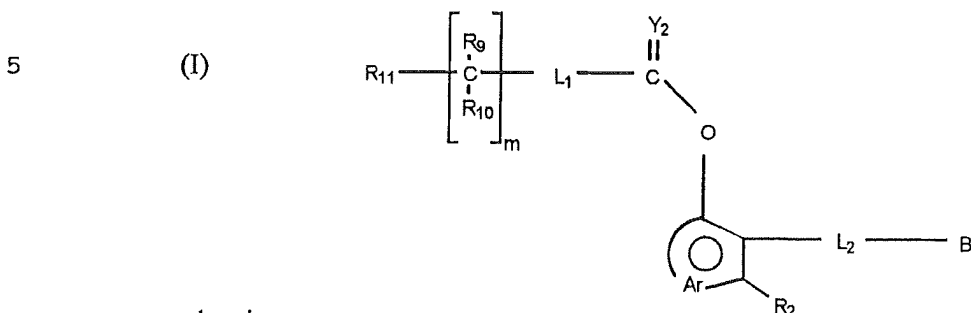
The problems associated with preparing prodrugs of amine-containing drugs was recently highlighted by Shan, D. et al. in "Prodrug Strategies Based on Intramolecular Cyclization Reactions" J. Pharm. Sci. July 1997 Vol.86, No.7, 765-767, (the contents of which are hereby incorporated by reference). To avoid the relative stability of the amide bond, the authors disclose prodrugs which incorporate various moieties which are capable of undergoing intramolecular cyclization reactions to release the parent drug. The chemical or biological triggering mechanisms which initiate the cyclization reactions are independent of those which are required for releasing the original drug via hydrolysis of the amide bond. Again, non-polymeric-

based systems are disclosed.

It is believed that in spite of the reported work in the field of double prodrugs, some specific problems were not addressed sufficiently. For example, the previously reported techniques do not sufficiently address the solubility problems of many parent compounds. In addition, the problem of designing in a sufficient increase in circulating half-life for the prodrug was also not sufficiently developed. Thus, there continues to be a need to provide additional technologies for forming prodrugs which would benefit from the double prodrug concept. For example, it would be advantageous to provide the artisan with alternative techniques for transport carrier attachment so as to regulate biological effect. Furthermore, it would be desirable to provide additional techniques to address problems associated with involving amino residues and/or hydroxyl residues of parent compounds and thus avoid excessively fast or slow hydrolysis of the transport form from the parent compound at physiological pH.

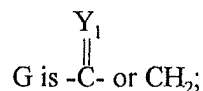
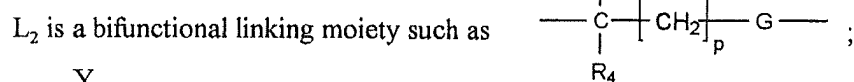
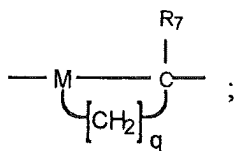
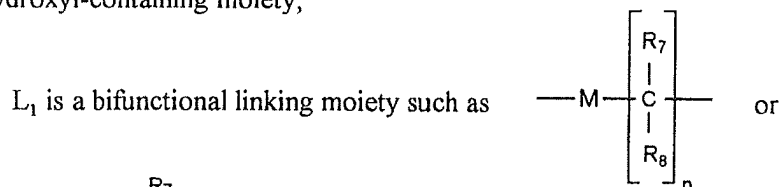
SUMMARY OF THE INVENTION

The present invention addresses the shortcomings described above. In one aspect of the invention, compounds of Formula (I) are provided:



wherein:

B is H, OH, OSiR₁₃, a residue of an amine-containing target moiety or a residue of a hydroxyl-containing moiety;



Y_{1,2} are independently O or S;

M is X or Q; wherein

X is an electron withdrawing group

Q is a moiety containing a free electron pair positioned three to six atoms from C(=Y₂);

R₁, R₂, and R₄ are independently one of C₁₋₆ alkyls, C₃₋₁₂ branched alkyls, C₃₋₈

cycloalkyls, C₁₋₆ substituted alkyls, C₃₋₈ substituted cycloalkyls, aryls, substituted aryls, aralkyls, C₁₋₆ heteroalkyls, substituted C₁₋₆ heteroalkyls, C₁₋₆ alkoxy, phenoxy, and C₁₋₆ heteroalkoxy;

R₇, R₈, R₉, R₁₀ and R₁₃ are independently hydrogen or a member of the group which defines R₁, R₂, and R₄;

Ar is a moiety which when included in Formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group;

(m) is zero or one;

(n) is zero or a positive integer;

(p) is zero, one or two;

(q) is three or four; and

R₁₁ is a polymer residue, such as a water-soluble polyalkylene oxide.

In certain preferred aspects, Ar is a di-alkyl substituted phenyl such as a dimethylphenyl and/or B is a leaving group such as N-hydroxybenzotriazolyl, N-hydroxyphthalimidyl, halogen, *p*-nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, thiazolidyl thione, or other activating groups. Alternatively, B is a residue of any amino-containing or hydroxyl-containing compound for which one or more of improved aqueous solubility, decreased antigenicity, prodrug and/or controlled release delivery is desired. For example, B, can be a residue of an enzyme, protein, or organic compound such as daunorubicin, doxorubicin, *p*-aminoaniline mustard, camptothecin, paclitaxel, AraC, etc.

For purposes of the present invention, the term "residue" shall be understood to mean that portion of a biologically active compound which remains after it has undergone a reaction in which the prodrug carrier portion has been attached by modification of a hydroxyl or amino group.

For purposes of the present invention, the term "alkyl" shall be understood to include straight, branched, substituted C₁₋₁₂ alkyls, including alkoxy, C₃₋₈ cycloalkyls or

substituted cycloalkyls, etc.

The double prodrugs of the present invention are thus unique delivery systems. Preferably the polymeric portion is first released by hydrolysis or esterase activity and then the resultant "second prodrug" moiety undergoes a cyclization reaction to regenerate the amine-or hydroxyl-containing parent (i.e. bioactive) compound in vivo.

Some of the chief advantages of the double prodrug compounds of the present invention are that they are capable of solubilizing amine-or hydroxyl-containing compounds and extending their half-life as compared to the native or even "second" prodrug counterparts. The polymeric portion can also impart an antigenicity-reducing effect on the parent compound. Another advantage of the systems of the present invention is that the linkage between the polymer portion and the "second prodrug" compound as described above, is designed to hydrolyze or otherwise cleave at a rate which allows the compound to retain its enhanced solubility and circulating half-life. The native drug, however, is still not released at this point. Only after the "second prodrug" undergoes the relatively rapid trialkyl lock lactonization reaction will the desired native or parent molecule be released. It is readily apparent that this double prodrug approach of the present invention offers unique and unexpected characteristics which enhance the circulating half-life and solubility of native or unmodified molecules.

Methods of making and using the compounds and conjugates described herein are also provided.

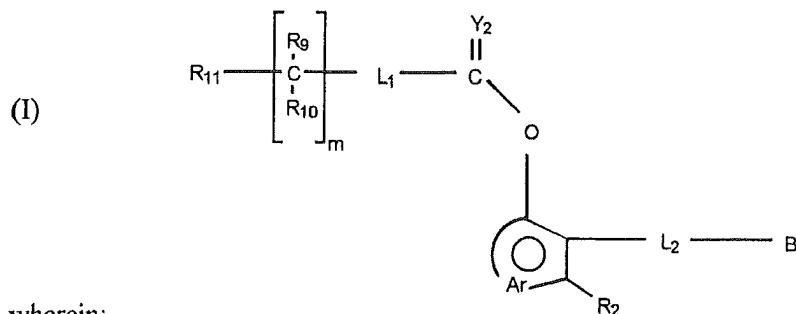
BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-2 schematically illustrate two methods of forming double prodrugs of the present invention.

Figures 3-16 illustrates the reaction schemes associated with the Examples.

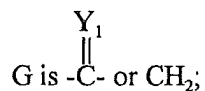
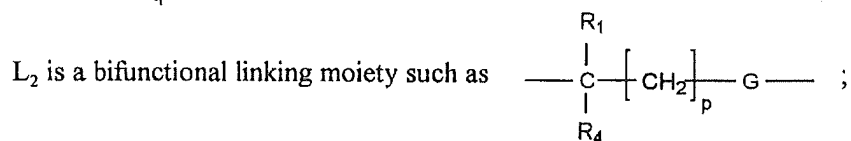
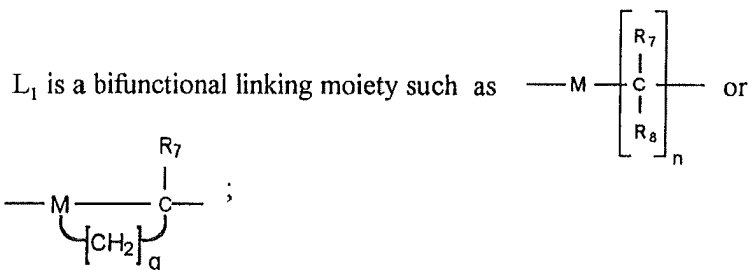
DETAILED DESCRIPTION OF THE INVENTION**A. FORMULA (I)**

In one aspect of the invention, there are provided compounds of formula (I):



wherein:

B is H, OH, OSiR₁₃, a residue of an amine-containing target moiety or a residue of a hydroxyl-containing moiety;



Y₁₋₂ are independently O or S;

M is X or Q; wherein

X is an electron withdrawing group

Q is a moiety containing a free electron pair positioned three to six atoms from C(=Y₂);

R_1 , R_2 , and R_4 are independently one of C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy, and C_{1-6} heteroalkoxy;

5 R_7 , R_8 , R_9 , R_{10} and R_{13} are independently one of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy, and C_{1-6} heteroalkoxy;

10 Ar is a moiety which when included in Formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group;

(m) is zero or one;

(n) is zero or a positive integer;

(p) is zero, one or two;

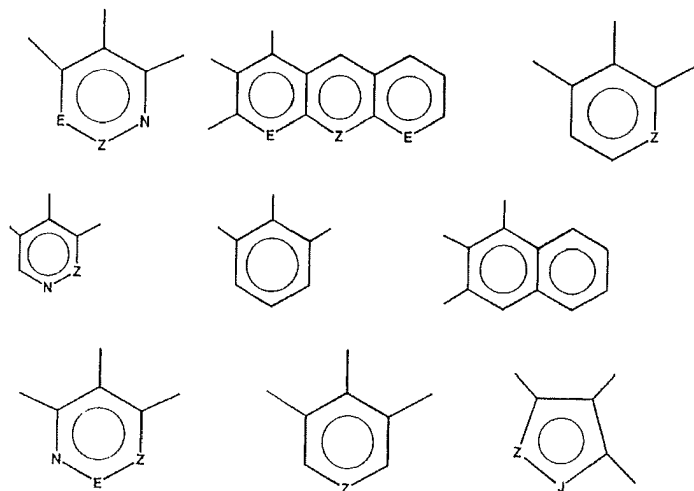
(q) is three or four; and

15 R_{11} is a polymer residue.

B. DESCRIPTION OF THE Ar MOIETY

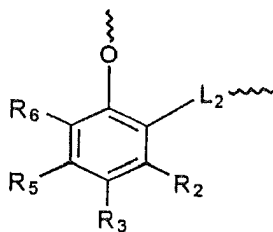
Referring to Formula (I), it can be seen that the Ar moiety is a moiety which when included in Formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group. A key feature is that the Ar moiety is aromatic in
20 nature. Generally, to be aromatic, the π electrons must be shared within a "cloud" both above and below the plane of a cyclic molecule. Furthermore, the number of π electrons must satisfy the Hückle rule ($4n+2$). Those of ordinary skill will realize that a myriad of moieties will satisfy the aromatic requirement of the moiety for Formula (I) and thus are suitable for use herein.

25 Preferred aromatic hydrocarbon moieties include, without limitation:



wherein J is O, S, or NR_{14} , E and Z are independently CR_{14} or NR_{14} ; and R_{14} is selected from the same group as that which defines R_9 in Formula (I) e.g. hydrogen, C_{1-6} alkyls, etc. Isomers of the five and six-membered rings are also contemplated as well as benzo- and dibenzo- systems and their related congeners are also contemplated. It will also be appreciated by the artisan of ordinary skill that the aromatic rings can optionally be substituted with heteroatoms such as O, S, NR_{14} , etc. so long as Hückel's rule is obeyed. Furthermore, the aromatic or heterocyclic structures may optionally be substituted with halogen(s) and/or side chains as those terms are commonly understood in the art. However, all structures suitable for Ar moieties of the present invention are capable of allowing the L_2 and R_2 moieties to be in an ortho arrangement within the same plane, as shown below:

(Ar-I)



where L_2 and R_2 are as defined above with regard to Formula (I) and R_3 , R_5 , and R_6 are independently selected from the group which defines R_9 or a cyano, nitro, carboxyl, acyl, substituted acyl, carboxyalkyl.

In preferred aspects of the present invention, R_2 is a C_{1-6} alkyl. More preferably, R_2 is methyl. Furthermore, R_1 and R_4 are preferably alkyl, e.g. methyl. Y_{1-2} are preferably O, and (p) is preferably one.

For purposes of the present invention, substituted alkyls include carboxyalkyls, aminoalkyls, dialkylaminos, hydroxyalkyls and mercaptoalkyls; substituted cycloalkyls include moieties such as 4-chlorocyclohexyl; aryls include moieties such as naphthyl; substituted aryls include moieties such as 3-bromophenyl; aralkyls include moieties such as toluyl; heteroalkyls include moieties such as ethylthiophene; substituted heteroalkyls include moieties such as 3-methoxy-thiophene; alkoxy includes moieties such as methoxy; and phenoxy includes moieties such as 3-nitrophenoxy. Halo- shall be understood to include fluoro, chloro, iodo and bromo.

C. LINKER MOIETIES L_1 AND L_2

As shown above, the invention includes bifunctional linking moieties L_1 and L_2 , with L_1 serving to join R_{11} and $C(=Y_2)$, and L_2 joining the Ar moiety and B. In preferred embodiments, L_1 includes the moiety (M) which is either an electron withdrawing group (designated herein as X), or a moiety containing a free electron pair positioned three to six atoms from the $C(=Y_2)$ (designated herein as Q). L_1 and L_2 can also include bifunctional moieties such as C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, etc.

D. THE DOUBLE PRODRUG LINKAGE PORTION

The first labile bond of the double prodrug system, which joins the L_1 to $\overset{Y_2}{\underset{\parallel}{C}}$, is selected to hydrolyze, such as via an esterase catalyzed hydrolysis *in vivo* at a rate which generates sufficient amounts of the "second" prodrug compound within a

When present as part of L₁, (n) is preferably 1 or 2.

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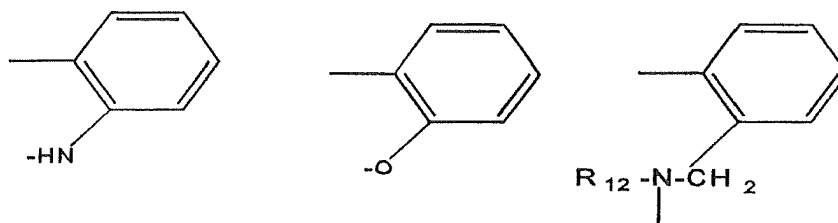
2. Q Portion of the Linker

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-CH₂-C(=O)-NH-, and ortho-substituted phenyls such as



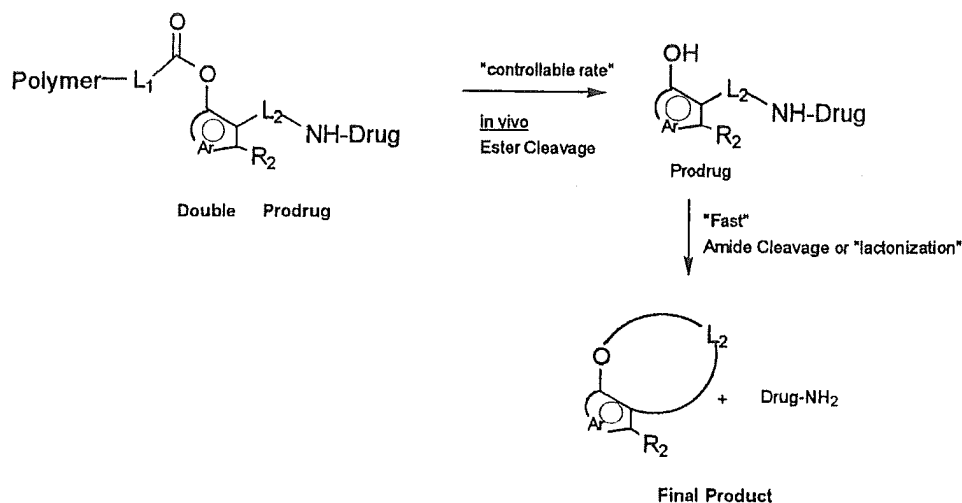
3. Drug Generation Via Hydrolysis of the Prodrug

The prodrug compounds of the present invention are designed so that $t_{1/2}$ of hydrolysis < $t_{1/2}$ elimination in plasma.

5 The linkages included in the compounds have hydrolysis rates in the plasma of the mammal being treated which is fast enough to allow sufficient amounts of the parent compounds, i.e. the amino-or hydroxyl-containing bioactive compound, to be released prior to elimination. Some preferred compounds of the present invention, i.e. those in which (*n*) is 1, have a $t_{1/2}$ for hydrolysis in plasma ranging from about 5
10 minutes to about 12 hours. Preferably, the compositions have a plasma $t_{1/2}$ hydrolysis ranging from about 0.5 to about 8 hours and most preferably from about 1 to about 6 hours.

4. Lactonization and Native Drug Regeneration

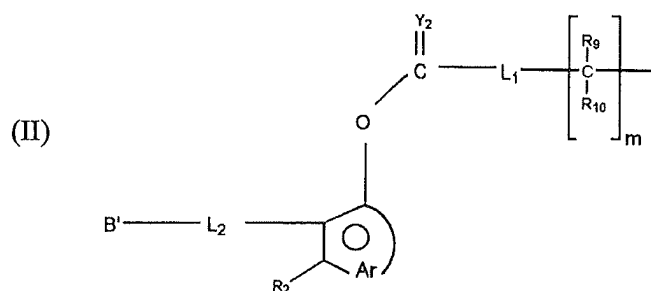
15 Once the first ester hydrolysis of the double prodrug has taken place in vivo, usually via esterase activity or pH moderated activity or cyclization reaction, the polymeric residue is cleaved and resultant second prodrug moiety remains. This single prodrug entity then undergoes a further independent lactonization reaction in vivo to produce the desired native or parent compound. This spontaneous reaction occurs after the rate controlling first hydrolysis step and is initiated by simple proton removal
20 of aromatic intermediate which causes a lactonization reaction and release of the parent molecule. The reaction is shown below.



Release of a hydroxyl-containing parent compound would occur in a similar fashion.

E. SUBSTANTIALLY NON-ANTIGENIC POLYMERS

The "double prodrug" compositions of the present invention include a water-soluble polymer, R₁₁. In preferred aspects of the invention, R₁₁ includes a capping group A which can be hydrogen, C₁₋₆ alkyl moieties, carboxyalkyl, dialkyl acyl urea alkyls, or a compound of formula (II) shown below which forms a bis-system:



wherein B' is the same as B or another member of the group defined by B and the remaining variables are as set forth above with regard to Formula (I).

Suitable examples of such polymers include polyalkylene oxides such as polyethylene glycols which are also preferably substantially non-antigenic. The general formula for PEG and its derivatives, i.e. $A'-O-(CH_2CH_2O)_x-(CH_2)_n-A$, where (x) represents the degree of polymerization (i.e. 10-2,300) or number of repeating units in the polymer chain and is dependent on the molecular weight of the polymer; (n) is zero or a positive integer; A is a capping group as defined herein, i.e. an -H, amino, carboxy, halo, C_{1-6} alkyl or other activating group and A' is the same as A or another A moiety. Also useful are polypropylene glycols, branched PEG derivatives such as those described in commonly-assigned U.S. Patent No. 5,643,575, "star-PEG's" and multi-armed PEG's such as those described in Shearwater Polymers, Inc. catalog "Polyethylene Glycol Derivatives 1997-1998"; the disclosure of each is incorporated herein by reference. It will be understood that the water-soluble polymer will be functionalized for attachment to the linkage via M, X or Q herein. As an example, the PEG portion of the prodrugs can be the following non-limiting compounds:

$-C(=Y)-(CH_2)_n-O-(CH_2CH_2O)_x-A$, $-C(=Y)-Y-(CH_2)_n-O-(CH_2CH_2O)_x-A$ and $-C(=Y)-NR_{12}-(CH_2)_n-O-(CH_2CH_2O)_x-A$, where Y is O or S and R_{12} , (n) and (x) are as defined above.

In many aspects of the invention, polyethylene glycols (PEG's), mono-activated, C_{1-4} alkyl-terminated PAO's such as mono-methyl-terminated polyethylene glycols (mPEG's) are preferred.

In order to provide the desired hydrolyzable linkage, mono- or di-acid activated polymers such as PEG acids or PEG diacids can be used as well as mono- or di-PEG amines and mono- or di-PEG diols. Suitable PAO acids can be synthesized by first converting mPEG-OH to an ethyl ester followed by saponification. See also Gehrhardt, H., et al. Polymer Bulletin 18: 487 (1987) and Veronese, F.M., et al., J. Controlled Release 10: 145 (1989). Alternatively, the PAO-acid can be synthesized by converting mPEG-OH into a *t*-butyl ester followed by acid cleavage. See, for example,

commonly assigned U.S. Patent No. 5,605,976. The disclosures of each of the foregoing are incorporated by reference herein.

Although PAO's and PEG's can vary substantially in number average molecular weight, polymers ranging from about 2,000 to about 100,000 are usually selected for the purposes of the present invention. Molecular weights of from about 5,000 to about 50,000 are preferred and 5,000 to about 40,000 are particularly preferred. The number average molecular weight of the polymer selected for inclusion in the "double prodrug" must be sufficient so as to provide sufficient circulation of the "double prodrug" before hydrolysis of the linker. Within the ranges provided above, polymers having molecular weight ranges of at least 20,000 are preferred in some aspects for chemotherapeutic and organic moieties. In the case of some nucleophiles such as certain proteins, enzymes and the like, polymers having a molecular weight range of from about 2,000 to about 20,000 are preferred.

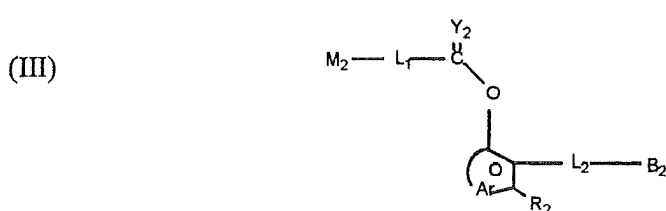
The polymeric substances included herein are preferably water-soluble at room temperature. A non-limiting list of such polymers include polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof, provided that the water solubility of the block copolymers is maintained.

As an alternative to PAO-based polymers, effectively non-antigenic materials such as dextran, polyvinyl alcohols, carbohydrate-based polymers, polyaminoacids hydroxypropylmethacrylamide (HPMA), and copolymers thereof etc. and the like can be used if the same type of activation is employed as described herein for PAO's such as PEG. Those of ordinary skill in the art will realize that the foregoing list is merely illustrative and that all polymeric materials having the qualities described herein are contemplated. For purposes of the present invention, "effectively non-antigenic" means all polymeric materials understood in the art as being nontoxic and not eliciting an appreciable immune response in mammals.

F. POLYMERIC DOUBLE PRODRUG TRANSPORT SYSTEM SYNTHESIS

The double prodrugs of the present invention can be prepared in at least two fashions. One technique schematically shown in Figure 1, includes

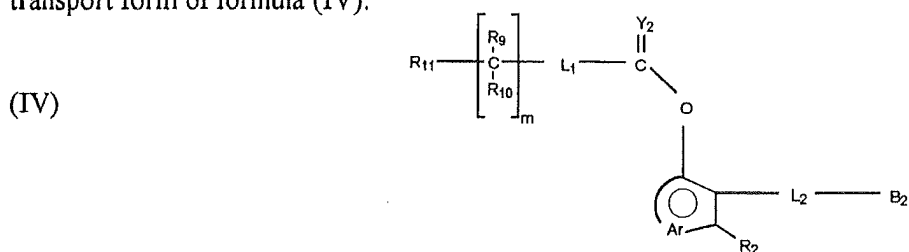
- a. providing an intermediate compound (III)



where M_2 is a cleavable or reversible protecting group, B_2 is a leaving group such as OH, and all other variables are as set forth above with regard to Formula (I);

- b. treating the intermediate compound (III) with a strong acid such as TFA (trifluoroacetic acid) or other trihaloacetic acid, HCl, sulfuric acid, etc., or catalytic hydrogenation to remove the protecting group; and

- c. reacting the unprotected intermediate compound (III) obtained by step b. with a moiety capable of reacting with L_1 , such as an activated polymer, i.e. a polymer having a reactive functional group, (designated R_{11} , in Figure 1) e.g., p-nitrophenyl or succinimidyl carbonate, carbonyl imidazole, thiazolidinyl thione or the like, and optional spacer group, i.e. CR_9R_{10} , to form an activated double prodrug transport form of formula (IV):



where B_2 is a leaving group and all other variables are as set forth above with regard to Formula (I); and optionally

d. attaching an amine-containing or hydroxyl-containing compound residue, e.g. the drug to be transported, to compound (IV) by displacing B₂ in a reaction with an amine-or hydroxyl-containing compound, See Figure 1. Similar techniques are employed when other aromatic moieties are used as starting materials.

5 Alternatively, as shown in Figure 2 with an amine-containing compound for illustrative purposes, the double prodrug can be prepared by:

- a. attaching an amine-containing or hydroxyl-containing compound to the intermediate compound (III);
- b. removing the protecting group; and
- 10 c. reacting the unprotected intermediate with an activated polymer to form the double prodrug.

Although not illustrated in Figure 2, the reaction scheme for an OH-containing compound, the reaction scheme would nonetheless proceed in the manner of Figure 2 with an ester bond being formed between the transport system and drug residue.

15 Intermediate compound (III) can be prepared using standard organic synthesis techniques. For example, blocked dialkylphenyl propionic acid derivatives such as 3-(2'-Boc-alanyl-4',4'-dimethylphenyl)-3,3-dimethylpropionic acid can be synthesized using a procedure disclosed by Binghe Wang, et al. Synthesis of a Novel Esterase-Sensitive Cyclic Prodrug System for Peptides That Utilizes a "Trimethyl Lock" -
20 Facilitated Lactonization Reaction. *J. Org. Chem.* 1997, 62, 1363.), the disclosure of which is incorporated herein by reference. Alternative aromatic starting materials will be apparent to those of ordinary skill without undue experimentation.

Attachment of the parent compound to intermediate compound (III) or (IV) can be carried out using standard organic synthesis techniques using coupling agents
25 known to those of ordinary skill in the art such as 1,3-diisopropylcarbodiimide (DIPC), dialkyl carbodiimides, 2-halo-1-alkyl-pyridinium halides, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), propane phosphonic acid cyclic anhydride (PPACA) and

phenyl dichlorophosphates. Alternatively, when B is a good leaving group such as those listed below in G.1., a coupling agent is not required and the reaction proceeds in the presence of a base.

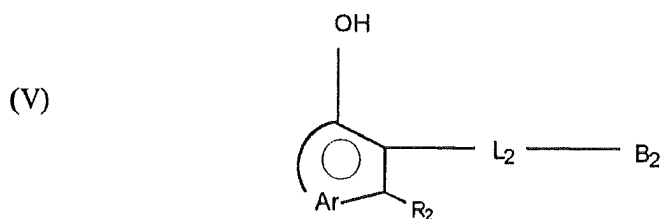
Removal of the protecting group is carried out in the same manner as described in the first method.

G. THE LEAVING GROUP OR RESIDUE PORTION "B"

1. Leaving Groups

In those aspects where B is a leaving group, suitable leaving groups include, without limitations, moieties such as N-hydroxybenzotriazolyl, halogen, N-hydroxyphthalimidyl, *p*-nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl; thiazolidinyl thione, or other good leaving groups as will be apparent to those of ordinary skill.

The leaving groups can be attached to the L₂ portion of the compound after the "double" prodrug portion, i.e. the PEG portion has been formed. The synthesis reactions used and described herein will be understood by those of ordinary skill without undue experimentation. For purposes of illustration and not limitation, generally, the activated forms of the double prodrug transport system are prepared by acylating the aromatic hydroxy of a compound of formula (V) below:



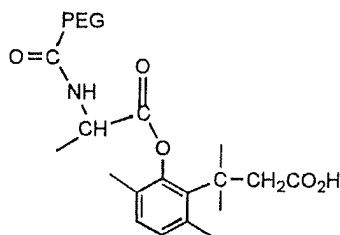
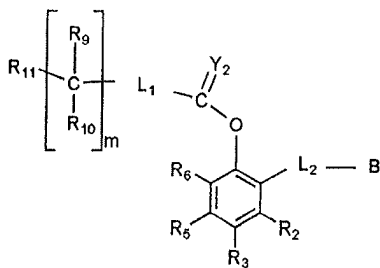
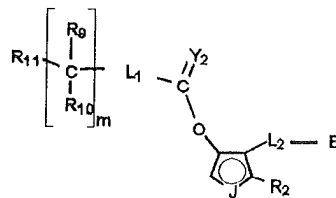
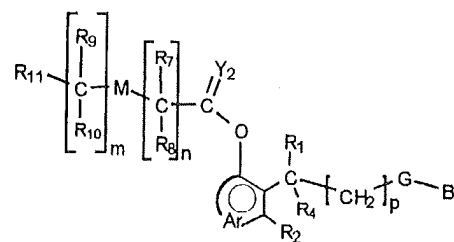
wherein:

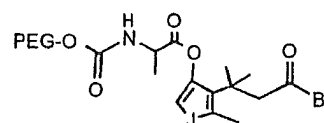
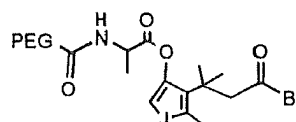
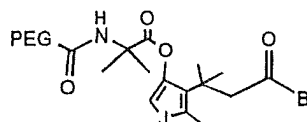
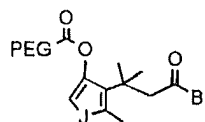
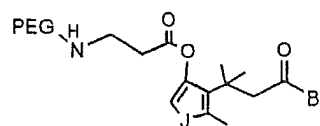
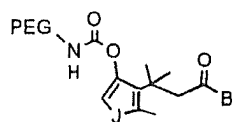
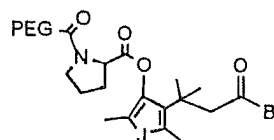
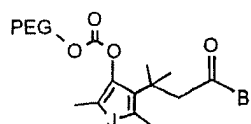
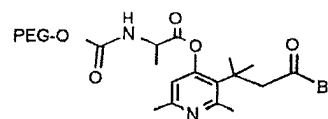
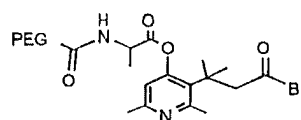
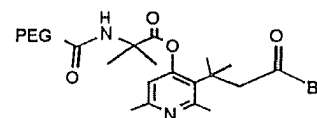
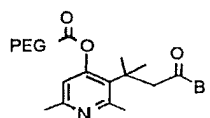
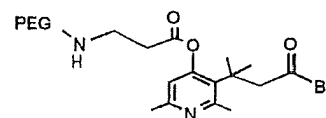
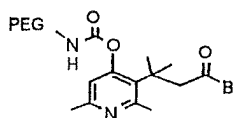
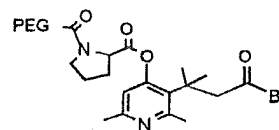
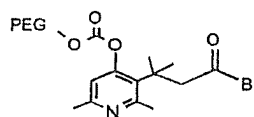
L₂ and B₂ are as set forth above and thereafter reacting the acylated resultant compound with an activating group for coupling to a target, e.g., compounds such as

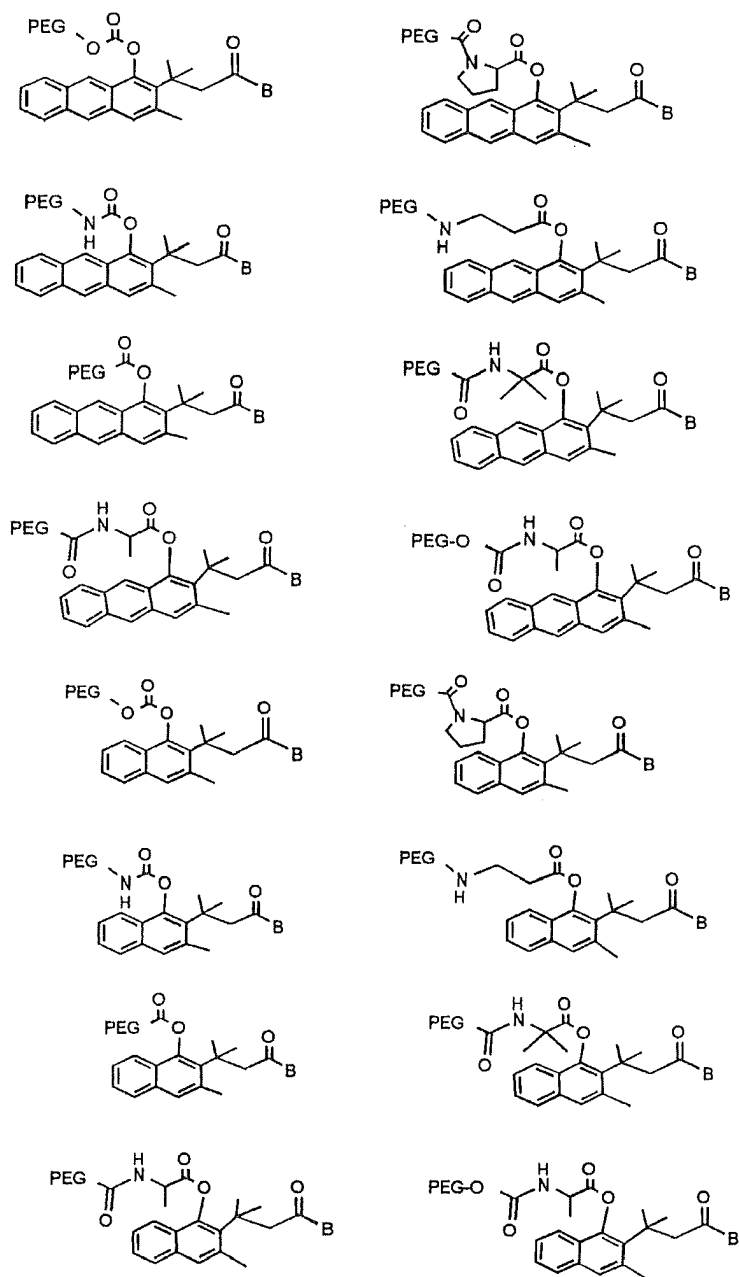
4-nitrophenyl-chloroformate, disuccinimidyl carbonate (DSC), carbonyldiimidazole, thiazolidine thione, etc. to provide the desired derivative.

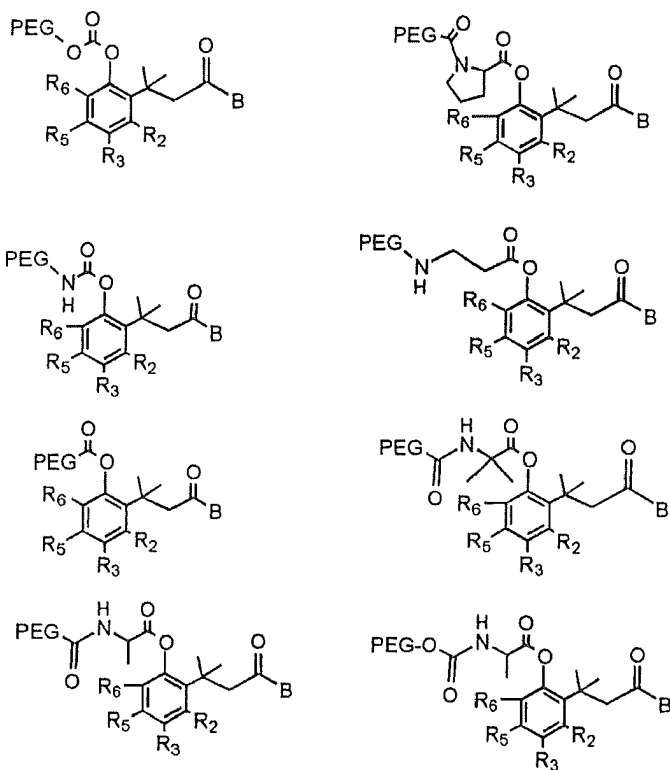
Once in place, the "activated" form of the PEG prodrug is ready for conjugation with an amine- or hydroxyl-containing compound. Some preferred activated transport forms are shown below:

5









2. Residues of Amine-containing Compounds

In those aspects of the invention, e.g. after the prodrug transport has been formed, B is a residue of an amine-containing compound, a non-limiting list of such suitable compounds include residues of organic compounds, enzymes, proteins, polypeptides, etc. Organic compounds include, without limitation, moieties such as anthracycline compounds including daunorubicin, doxorubicin; *p*-aminoaniline mustard, Ara-C (cytosine arabinoside) and related anti-metabolite compounds, e.g., gemcitabine, etc. Alternatively, B can be a residue of an amine-containing cardiovascular agent, anti-neoplastic, anti-infective, anti-fungal such as nystatin and amphotericin B, anti-anxiety agent, gastrointestinal agent, central nervous system-activating agent,

analgesic, fertility agent, contraceptive agent, anti-inflammatory agent, steroidal agent, anti-urecemic agent, vasodilating agent, vasoconstricting agent, etc.

Suitable proteins, polypeptides, enzymes, peptides and the like having at least one available amino group for polymer attachment include materials which have physiological or pharmacological activities as well as those which are able to catalyze reactions in organic solvents. The only other requirement of the amine-containing materials is that they maintain at least some portion of the activity associated with the unmodified protein, enzyme, peptide, etc. after the prodrug transport portion has hydrolyzed.

Proteins, polypeptides and peptides of interest include, but are not limited to, hemoglobin, serum proteins such as blood factors including Factors VII, VIII, and IX; immunoglobulins, cytokines such as interleukins, i.e. IL-1 through IL-13, α -, β - and γ - and consensus interferons, colony stimulating factors including granulocyte colony stimulating factors, platelet derived growth factors and phospholipase-activating protein (PLAP). Other proteins of general biological or therapeutic interest include insulin, plant proteins such as lectins and ricins, tumor necrosis factors and related proteins, growth factors such as transforming growth factors, such as TGF α 's or TGF β 's and epidermal growth factors, hormones, somatomedins, erythropoietin, pigmentary hormones, hypothalamic releasing factors, antidiuretic hormones, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, and the like. Immunoglobulins of interest include IgG, IgE, IgM, IgA, IgD and fragments thereof.

Some proteins such as the interleukins, interferons and colony stimulating factors also exist in non-glycosylated form, usually as a result of using recombinant techniques. The non-glycosylated versions are also among the proteins of the present invention.

Enzymes of interest include carbohydrate-specific enzymes, proteolytic

enzymes, oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Without being limited to particular enzymes, examples of enzymes of interest include asparaginase, arginase, arginine deaminase, adenosine deaminase, superoxide dismutase, endotoxinases, catalases, chymotrypsin, lipases, uricases, adenosine diphosphatase, tyrosinases and bilirubin oxidase. Carbohydrate-specific enzymes of interest include glucose oxidases, glucodases, galactosidases, glucocerebrosidases, glucouronidases, etc.

Also included herein is any portion of a polypeptide demonstrating in vivo bioactivity. This includes amino acid sequences, nucleic acids (DNA, RNA), peptide nucleic acids (PNA), antibody fragments, single chain binding proteins, see, for example U.S. Patent No. 4,946,778, disclosure of which is incorporated herein by reference, binding molecules including fusions of antibodies or fragments, polyclonal antibodies, monoclonal antibodies and catalytic antibodies.

The proteins or portions thereof can be prepared or isolated by using techniques known to those of ordinary skill in the art such as tissue culture, extraction from animal sources, or by recombinant DNA methodologies. Transgenic sources of the proteins, polypeptides, amino acid sequences and the like are also contemplated. Such materials are obtained from transgenic animals, i.e., mice, pigs, cows, etc., wherein the proteins are expressed in milk, blood or tissues. Transgenic insects and baculovirus expression systems are also contemplated as sources. Moreover, mutant versions of proteins, such as mutant interferons are also within the scope of the invention.

Other proteins of interest are allergen proteins such as ragweed, Antigen E, honeybee venom, mite allergen, and the like. The foregoing is illustrative of the proteins which are suitable for the present invention. It is to be understood that those proteins, as defined herein, not specifically mentioned but having an available amino group are also intended and are within the scope of the present invention.

In a preferred aspect of the invention, the amino-containing compound is a biologically active compound that is suitable for medicinal or diagnostic use in the treatment of animals, e.g., mammals, including humans, for conditions for which such treatment is desired. The foregoing list is meant to be illustrative and not limiting for the compounds which can be modified. Those of ordinary skill will realize that other such compounds can be similarly modified without undue experimentation. It is to be understood that those biologically active materials not specifically mentioned but having suitable amino-groups are also intended and are within the scope of the present invention.

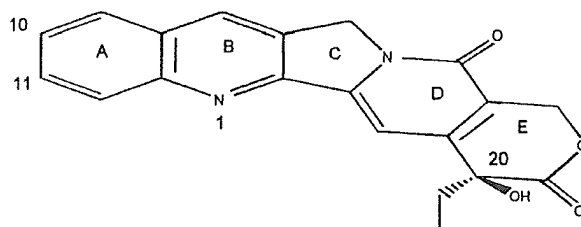
The only limitations on the types of amino-containing molecules suitable for inclusion herein is that there is available at least one (primary or secondary) amine containing position which can react and link with a carrier portion and that there is not substantial loss of bioactivity after the double prodrug system releases and regenerates the parent compound.

3. Residues of Hydroxyl-Containing Compounds

a. Camptothecin and Related Topoisomerase I Inhibitors

Camptothecin is a water-insoluble cytotoxic alkaloid produced by *Camptotheca accuminata* trees indigenous to China and *nothapodytes foetida* trees indigenous to India. Camptothecin and related compounds and analogs are also known to be potential anticancer or antitumor agents and have been shown to exhibit these activities in vitro and in vivo. Camptothecin and related compounds are also candidates for conversion to the double prodrugs of the present invention.

Camptothecin and certain related analogues share the structure:

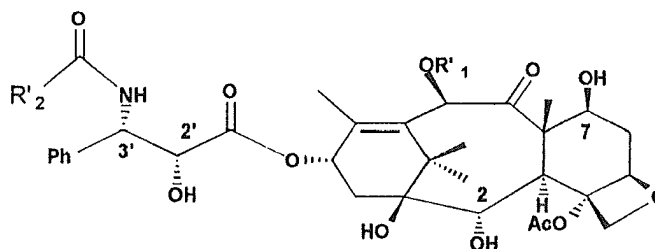


From this core structure, several known analogs have been prepared. For example, the A ring in either or both of the 10- and 11-positions can be substituted with an OH. The A ring can also be substituted in the 9-position with a straight or branched C₁₋₃₀ alkyl or C₁₋₁₇ alkoxy, optionally linked to the ring by a heteroatom i.e.- O or S. The B ring can be substituted in the 7-position with a straight or branched C₁₋₃₀ alkyl or substituted alkyl-, C₅₋₈ cycloalkyl, C₁₋₃₀ alkoxy, phenyl alkyl, etc., alkyl carbamate, alkyl carbazides, phenyl hydrazine derivatives, amino-, aminoalkyl-, aralkyl, etc. Other substitutions are possible in the C, D and E rings. See, for example, U.S. Patent Nos. 5,004,758; 4,943,579; Re 32,518, the contents of which are incorporated herein by reference. Such derivatives can be made using known synthetic techniques without undue experimentation. Preferred camptothecin derivatives for use herein include those which include a 20-OH or another OH moiety which is capable of reacting directly with activated forms of the polymer transport systems described

herein or to the linking moiety intermediates, e.g. iminodiacetic acid, etc., which are then attached to a polymer such as PEG. Reference to camptothecin analogs herein has been made for purposes of illustration and not limitation.

b. Taxanes and Paclitaxel Derivatives

One class of compounds included in the double prodrug compositions of the present invention is taxanes. For purposes of the present invention, the term "taxane" includes all compounds within the taxane family of terpenes. Thus, taxol (paclitaxel), 3'-substituted tert-butoxy-carbonyl-amine derivatives (taxoteres) and the like as well as other analogs which are readily synthesized using standard organic techniques or are available from commercial sources such as Sigma Chemical of St. Louis, Missouri are within the scope of the present invention. Representative taxanes are shown below.



Paclitaxel: $R'_1 = C_6H_5$; $R'_2 = CH_3CO$; Taxotere: $R'_1 = (CH_3)_3CO$; $R'_2 = H$

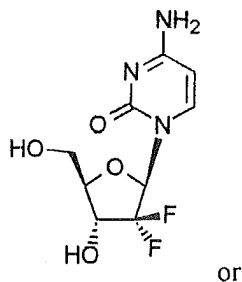
These derivatives have been found to be effective anti-cancer agents. Numerous studies indicate that the agents have activity against several malignancies. To date, their use has been severely limited by, among other things, their short supply, poor water solubility and hypersensitivity. It is to be understood that other taxanes including the 7-aryl-carbamates and 7-carbazates disclosed in commonly assigned U.S. Patent Nos. 5,622,986 and 5,547,981 can also be included in the double prodrugs of the present invention. The contents of the foregoing U.S. patents are incorporated

herein by reference. The only limitation on the taxane is that it must be capable of undergoing a hydroxyl based substitution reaction such as at the 2' position. Paclitaxel, however, is a preferred taxane.

c. Additional Biologically-Active Moieties

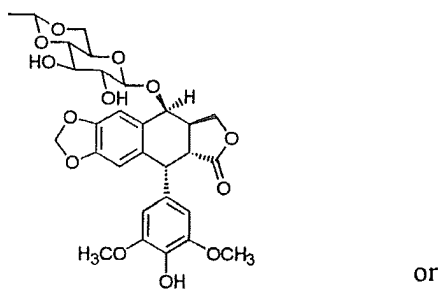
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In addition to the foregoing molecules, the double prodrug formulations of the present invention can be prepared using many other compounds. For example, biologically-active compounds such as gemcitabine:

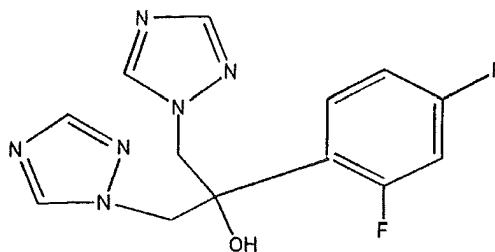


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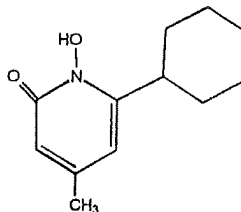
etoposide:



triazole-based antifungal agents such as fluconazole:



or ciclopirox:



can be used.

The parent compounds selected for double prodrug forms need not be substantially water-insoluble, although the polymer-based double prodrugs of the present invention are especially well suited for delivering such water-insoluble compounds. Other useful parent compounds include, for example, certain low molecular weight biologically active proteins, enzymes and peptides, including peptidoglycans, as well as other anti-tumor agents; cardiovascular agents such as forskolin; anti-neoplastics such as combretastatin, vinblastine, doxorubicin, Ara-C, maytansine, etc.; anti-infectives such as vancomycin, erythromycin, etc.; anti-fungals such as nystatin, amphotericin B, triazoles, papulocandins, pneumocandins, echinocandins, polyoxins, nikkomycins, pradimicins, benanomicins, etc. see, "Antibiotics That Inhibit Fungal Cell Wall Development" *Annu. Rev. Microbiol.* 1994, 48:471-97, the contents of which are incorporated herein by reference; anti-anxiety agents, gastrointestinal agents, central nervous system-activating agents, analgesics, fertility or contraceptive agents, anti-inflammatory agents, steroidal agents, anti-urecemic agents, cardiovascular agents, vasodilating agents, vasoconstricting agents and the like.

It is noted that parent compounds suitable for incorporation into the double prodrug compositions of the invention, may themselves be substances/compounds which are not active after hydrolytic release from the linked composition, but which will become active after undergoing a further chemical process/reaction. For example, an anticancer drug that is delivered to the bloodstream by the double prodrug transport system, may remain inactive until entering a cancer or tumor cell, whereupon it is activated by the cancer or tumor cell chemistry, e.g., by an enzymatic reaction unique

to that cell.

After conjugation, the remaining amine-or hydroxyl-containing compound is referred to as the residue of the unconjugated compound.

4. Polymeric Hybrids

5 In another aspect of the invention there are provided hybrid types of the polymeric double prodrug system described herein. In particular, the hybrid system includes not only the reversible double prodrug system but also a second polymeric system based on more permanent types of linkages. The hybrids can be prepared by at least two methods. For example, the trialkyl lock-based double prodrug protein
10 conjugate can be first synthesized, and then PEGylated using any art-recognized activated polymer such as thiazolidinyl thione or succinimidyl carbonate-activated PEG. Alternatively, the more permanent conjugation reaction can be performed first (i.e. the parent compound is PEGylated) and the resultant conjugates can be used to form the trialkyl lock double prodrug conjugates described herein. It will be
15 understood that the hybrid systems will be better suited for proteins, enzymes and the like where multiple amino groups or a combination of amino and hydroxyl groups are available for attachment of the polymeric prodrug. For purposes of the present invention, "activated polymers" will be understood to include polymers containing one or more terminal groups which are capable of reacting with one or more of α -amino
20 groups, ϵ -amino groups, histidine nitrogens, carboxyl groups, sulfhydryl groups, etc. found on enzymes, proteins, etc., as well as such groups found on synthetically prepared organic compounds.

The activating terminal moiety can be any group which facilitates conjugation of the polymers with the biologically active material, i.e. protein, enzyme, etc. either
25 before or after the double prodrug transport system of the present invention has been synthesized. See, for example, U.S. Patent No. 4,179,337, the disclosure of which is hereby incorporated by reference. Such activating groups can be a moiety selected

from:

- 5
- I. Functional groups capable of reacting with an amino group such as:
- a) carbonates such as the p-nitrophenyl, or succinimidyl; see, for example, U.S. Patent No. 5,122,614, the disclosure of which is hereby incorporated by reference;
 - b) carbonyl imidazole;
 - c) azlactones; see, for example, U.S. Patent No. 5,321,095, the disclosure of which is hereby incorporated by reference;
 - d) cyclic imide thiones see, for example, U.S. Patent No. 5,349,001, the disclosure of which is hereby incorporated by reference; or
 - e) isocyanates or isothiocyanates.
 - f) active esters such as N-hydroxy-succinimidyl or N-hydroxybenzotriazolyl.
- 10
- II. Functional groups capable of reacting with carboxylic acid groups and reactive carbonyl groups such as:
- a) primary amines; or
 - b) hydrazine and hydrazide functional groups such as the acyl hydrazides, carbazates, semicarbamates, thiocarbazates, etc.
- 15
- III. Functional groups capable of reacting with mercapto or sulfhydryl groups such as maleimides; see, for example, Shearwater Polymers Catalog "Polyethylene Glycol Derivatives 1997-1998", the disclosure of which is hereby incorporated by reference;
- 20
- IV. Functional groups capable of reacting with hydroxyl groups such as (carboxylic) acids or other nucleophiles capable of reacting with an electrophilic center, such as isocyanate, activated esters or carbonates, cyclic imides, thiones, etc.
- 25

The activating moiety can also include a spacer moiety located proximal to the

polymer. The spacer moiety may be a heteroalkyl, alkoxy, alkyl containing up to 18 carbon atoms or even an additional polymer chain. The spacer moieties can added using standard synthesis techniques.

H. METHODS OF TREATMENT

5 Another aspect of the present invention provides methods of treatment for various medical conditions in mammals. The methods include administering to the mammal in need of such treatment, an effective amount of a composition of the invention, as described herein, such as a double prodrug of doxorubicin. The prodrug compositions are useful for, among other things, treating diseases which are similar to
10 those which are treated with the parent compound, e.g. enzyme replacement therapy, neoplastic disease, reducing tumor burden, preventing metastasis of neoplasms and preventing recurrences of tumor/neoplastic growths in mammals.

 The amount of the prodrug that is administered will depend upon the amount of the parent molecule included therein. Generally, the amount of prodrug used in the
15 treatment methods is that amount which effectively achieves the desired therapeutic result in mammals. Naturally, the dosages of the various prodrug compounds will vary somewhat depending upon the parent compound, rate of *in vivo* hydrolysis, molecular weight of the polymer, etc. In general, double prodrug polymeric derivatives of nitrogen mustard derivatives are administered in amounts ranging from about 5 to
20 about 500 mg/m² per day. The range set forth above is illustrative and those skilled in the art will determine the optimal dosing of the prodrug selected based on clinical experience and the treatment indication. Actual dosages will be apparent to the artisan without undue experimentation.

 The compositions, including prodrugs, of the present invention can be included
25 in one or more suitable pharmaceutical compositions for administration to mammals. The pharmaceutical compositions may be in the form of a solution, suspension, tablet,

capsule or the like, prepared according to methods well known in the art. It is also contemplated that administration of such compositions may be by the oral and/or parenteral routes depending upon the needs of the artisan. A solution and/or suspension of the composition may be utilized, for example, as a carrier vehicle for injection or infiltration of the composition by any art known methods, e.g., by intravenous, intramuscular, subdermal injection and the like.

Such administration may also be by infusion into a body space or cavity, as well as by inhalation and/or intranasal routes. In preferred aspects of the invention, however, the prodrugs are parenterally administered to mammals in need thereof.

I. EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention. The underlined and bold-faced numbers recited in the Examples correspond to those shown in the Figures.

Example 1.

Compound 2: Trifluoroacetic Acid Salt of Alanine Ester of 3-(2'-Hydroxy-4',6'-dimethylphenyl)-3,3-dimethylpropionic Acid.

1 g (2.54 mmol) of *t*-Boc-alanine ester of 3-(2'-hydroxy-4',6'-dimethylphenyl)-3,3-dimethylpropionic acid, **1** (synthesized using the procedure of *J. Org. Chem.* 1997, 62, 1363.), was stirred in 20 mL of TFA-CH₂Cl₂ (1:1, v/v) at room temperature for 2 hours. Solvent was removed *in vacuo* and ether was added to the residue to precipitate **2** (0.6873 g, 67%).

¹H NMR (270 MHz, CDCl₃) δ 1.41 (3H, s, C(CH₃)₂), 1.42 (3H, s, C(CH₃)₂), 1.54 (3H, d, *J* = 8.1 Hz, CHCH₃), 2.21 (3H, s, Ar-CH₃), 2.45 (3H, s, Ar-CH₃), 2.65 (2H, s, CH₂COOH), 4.36 (1H, d, *J* = 8.1 Hz, CH-CH₃), 6.57 (1H, s, Ar-H), 6.81 (1H, s, Ar-H).

^{13}C NMR (67.80 MHz, CDCl_3) δ 15.27 (Ar- CH_3), 19.57 (Ar- CH_3), 24.66 (CHCH_3), 30.92 ($\text{C}(\text{CH}_3)_2$), 38.28 (CH_2COOH), 47.32 ($\text{C}(\text{CH}_3)_2$), 48.45 (CHCH_3), 122.23, 132.30, 133.97, 135.61, 138.05, 149.10 (Ar), 169.50 ($-\text{COOAr}$), 172.50 (COOH).

5

Example 2.**Compound 4: Coupling of 2 with SC PEG (5 kDa), 3.**

2 g (0.39 mmol) of SC PEG (5 kDa), **3**, was added to the mixture of 175.8 mg (0.6 mmol) of **2** and 280 μL (1.5 mmol) of *N,N*-diisopropylethylamine (DIPEA) in 40 mL of anhydrous dichloromethane (DCM). The reaction mixture was stirred at room temperature overnight. The solvent was removed and the product was crystallized from 80 mL of 2-propanol to give 1.86 g (93%) of **4** as a white solid.

10

^{13}C NMR (67.80 MHz, CDCl_3) δ 16.01 (Ar- CH_3), 18.94 (Ar- CH_3), 24.07 (CHCH_3), 30.11 ($\text{C}(\text{CH}_3)_2$), 30.20 ($\text{C}(\text{CH}_3)_2$), 37.49 (CH_2COOH), 46.04 ($\text{C}(\text{CH}_3)_2$), 49.11 (CHCH_3), 57.59 (OCH_3), 62.86-70.68 (PEG), 121.37, 131.04, 132.80, 134.54, 136.71, 148.62 (Ar), 154.80 ($\text{OC}(=\text{O})\text{NH}$), 170.82 ($-\text{COOAr}$), 171.27 (COOH).

15

Example 3.**Compound 6: Coupling of 4 with Daunorubicin Hydrochloride, 5.**

38.4 mg (0.2 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was added to the mixture of 0.5 g (0.1 mmol) of **4**, 84.6 mg (0.15 mmol) of daunorubicin hydrochloride, **5**, 40.4 mg (0.4 mmol) of *N*-methylmorpholine (NMM), and 20.3 mg (0.2 mmol) of 1-hydroxybenzotriazole hydrate (HOBT) in 10 mL of anhydrous DCM at 0 °C. The reaction mixture was stirred at room temperature overnight and filtered. The filtrate was concentrated *in vacuo* and the residue was recrystallized from 20 mL of 2-propanol to give 0.44 g (88%) of **6**. UV assay showed 0.7 equivalent (70% of coupling yield) of **5**.

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25

^{13}C NMR (67.80 MHz, CDCl_3) δ 15.66, 15.81, 18.96, 23.56, 24.45, 29.96, 30.78, 30.84, 32.32, 33.65, 38.61, 44.13, 17.78, 49.45, 55.48, 57.73, 63.17-75.35

(PEG), 99.79, 109.81, 109.94, 117.75, 117.80, 118.37, 119.26, 121.45, 131.39, 133.02, 133.21, 133.92, 134.74, 134.78, 134.85, 135.04, 137.30, 148.88, 154.36, 155.17, 155.41, 159.84, 169.36, 171.81, 185.14, 185.33, 210.58.

Example 4.

5 **Compound 8: Coupling of 2 with mPEG (5 kDa) Thiazolidine Thione, 7.**

0.5 g (0.1 mmol) of mPEG (5 kDa) thiazolidine thione was added to the mixture of 44 mg (0.15 mmol) of 2 and 70 μ L (0.4 mmol) of DIPEA in 10 mL of anhydrous DCM at room temperature. The mixture was stirred at room temperature overnight. Solvent was removed under reduced pressure and the residue was
10 recrystallized from 2-propanol to give 0.44 g (88%) of product as a white solid.

^{13}C NMR (67.80 MHz, CDCl_3) δ 16.22, 19.17, 24.29, 30.37, 37.71, 46.37, 47.29, 57.85, 67.40-72.38 (PEG), 121.58, 131.38, 132.95, 134.85, 136.99, 148.75, 169.08, 170.53, 171.50.

Example 5.

15 **Compound 9: Coupling of 8 with 5.**

39 mg (0.2 mmol) of EDC is added to the mixture of 0.5 g (0.1 mmol) of 8, 85 mg (0.15 mmol) of 5, 40 mg (0.4 mmol) of NMM, and 20 mg (0.2 mmol) of HOBT in 10 mL of anhydrous DCM at 0 °C. The reaction mixture is stirred at room temperature overnight and filtered. The filtrate is concentrated *in vacuo* and the residue
20 recrystallized from 20 mL of 2-propanol to give 0.45 g (90%) of 9.

Example 6.

Compound 11: Coupling of 2 with PEG (40 kDa) Dithiazolidine thione, 10.

1 g (0.025 mmol) of PEG (40 kDa) dithiazolidine thione, 10 was added to the mixture of 29 mg (0.099 mmol) of 2 and 36.6 μ L (0.20 mmol) of DIPEA in 15 mL of
25 anhydrous DCM. The mixture was stirred at room temperature overnight. Solvent was removed under reduced pressure and the residue was recrystallized from 2-propanol to give 0.8 g (80%) of 11 as a white solid.

^{13}C NMR (67.80 MHz, CDCl_3) δ 16.90, 19.48, 24.45, 30.81, 38.34, 46.83, 47.87, 68.52-72.82 (PEG), 76.19, 77.83, 122.00, 131.77, 133.76, 135.40, 137.51, 149.46, 169.28, 170.88, 171.55.

Example 7.

5 **Compound 12: Coupling of 11 with 5.**

94.5 mg (0.492 mmol) of EDC was added to the mixture of 2.5 g (0.0615 mmol) of **11** (40 kDa), 208 mg (0.3688 mmol) of **5**, 99.4 mg (0.984 mmol) of NMM, and 49.8 mg (0.369 mmol) of HOBT in 50 mL of anhydrous DCM at 0 °C. The reaction mixture was stirred at room temperature overnight and filtered. The filtrate
10 was concentrated *in vacuo* and the residue was recrystallized from 2-propanol (200 mL) to give 2.3 g (92%) of **12**. UV assay showed 1.9 equivalent (95% of coupling yield) of **5**.

^{13}C NMR (67.80 MHz, CDCl_3) δ 16.14, 19.31, 23.55, 24.63, 28.80, 31.15, 32.78, 34.77, 39.23, 44.97, 48.12, 56.18, 66.80-75.95 (PEG), 100.14, 110.93, 118.68,
15 119.13, 121.81, 131.88, 133.86, 134.02, 134.96, 135.03, 137.96, 149.56, 155.20, 160.73, 169.73, 170.22, 171.34, 186.05, 210.41.

Example 8.

Compound 14: Coupling of 11 with *p*-Amino-(*N,N*-di-2-chloroethyl)aniline hydrochloride, 13.

20 37.8 mg (0.20 mmol) of EDC was added to the mixture of 1.0 g (0.025 mmol) of **4** (40 kDa), 39.8 mg (0.15 mmol) of *p*-amino-(*N,N*-di-2-chloroethyl)aniline hydrochloride, **13** (synthesized using a modified procedure of Edwards et al. Cytotoxic Compounds. Part XVII. *o*-, *m*-, and *p*-(Bis-2-chloroethylamino)phenol, *p*-[*N*-(2-Chloethyl)methylamino]phenol, *N,N*-Bis-2-chloroethyl-*p*-phenylenediamine, and *N,N*-
25 Bis-2-chloroethyl-*N*-methyl-*p*-phenylenediamine as Sources of Biologically Active Carbamates. *JCS Perkin I*, 1973, 2397.), 39.7 mg (0.39 mmol) of NMM, and 19.9 mg (0.15 mmol) of HOBT in 20 mL of anhydrous DCM at 0 °C. The reaction mixture was

stirred at room temperature overnight and filtered. The filtrate was concentrated *in vacuo* and the residue was recrystallized from 2-propanol (100 mL) to give 0.8 g (80%) of **14**. UV assay showed 1.5 equivalent (75% of coupling yield) of the aromatic nitrogen mustard.

¹³C NMR (67.80 MHz, CDCl₃) δ 15.63, 19.35, 24.86, 30.99, 31.10, 39.12, 39.81, 47.99, 48.36, 52.84, 67.79-71.79 (PEG), 111.59, 120.97, 121.57, 129.04, 131.86, 133.83, 135.38, 137.93, 141.53, 149.12, 168.37, 170.25, 171.66.

Example 9.

Compound 15: Coupling of **11 with 2-Mercaptothiazoline.**

3 g (0.074 mmol) of **11** was azeotroped in 60 mL of toluene by distillation of 20 mL of toluene for 2 hours. The solution was cooled to 35 °C and 47 mg (0.37 mmol) of oxalyl chloride and 2 µL of DMF were added. The solution was stirred at 35-40 °C for 4 h followed by addition of 53 mg (0.44 mmol) of 2-mercaptothiazoline and 32 mg (0.44 mmol) of DIPEA. The mixture was refluxed overnight and concentrated *in vacuo*. The residue was recrystallized from 250 mL of 2-propanol to give the **15** (2.72 g, 90%) as a white solid.

¹³C NMR (67.80 MHz, CDCl₃) δ 16.38, 19.28, 24.63, 27.54, 31.06, 31.15, 39.00, 47.37, 51.56, 55.32, 67.48-71.10 (PEG), 121.52, 131.43, 132.63, 134.86, 137.20, 148.79, 168.82, 170.90, 171.86, 200.58.

Example 10.

Compound 17: Coupling of **11 or **15** with Doxorubicin Hydrochloride, **16**.**

*Coupling of **11** with doxorubicin, **16**:* A mixture of 1.25 g (0.031 mmol) of **11**, 105 mg (0.18 mmol) of doxorubicin HCl, **16**, 50 mg (0.50 mmol) of NMM, 25 mg (0.18 mmol) of HOBT, and 50 mg (0.25 mmol) of EDC in 15 mL of anhydrous DCM was stirred at room temperature overnight. The solvent was removed *in vacuo* and the residue was recrystallized from 2-propanol to give 850 mg (68 %) of **17**. UV assay showed 1.64 equivalent (82% of coupling yield) **16**.

Coupling of 15 with 16: A mixture of 0.25 g (0.006 mmol) of **15**, 40 mg (0.07 mmol) of **16**, and 25 mg (0.20 mmol) of dimethylaminopyridine (DMAP) in 10 mL of anhydrous DCM was refluxed overnight. The solvent was removed *in vacuo* and the residue was recrystallized from 2-propanol to give 0.23 g (92%) of **17**. UV assay showed 1.82 equivalent (91% of coupling yield) of **16**.

¹³C NMR (67.80 MHz, CDCl₃) δ 15.21, 15.83, 18.75, 24.39, 27.81, 30.60, 30.63, 32.27, 34.80, 38.71, 43.90, 46.71, 47.87, 55.48, 64.27, 66.05, 67.43-71.05 (PEG) 75.69, 99.38, 109.47, 117.46, 118.68, 118.92, 120.45, 127.10, 131.70, 133.17, 132.89, 133.45, 133.75, 134.50, 137.44, 148.88, 154.41, 154.81, 159.69, 169.16, 170.15, 170.98, 185.20, 185.46, 212.40.

Example 11.

Compound 18: Coupling of 8 with *N*-Hydroxysuccinimide.

2.5 g (0.47 mmol) of **8** and 108 mg (0.94 mmol) of *N*-hydroxysuccinimide were dissolved in 40 mL of anhydrous DCM at 0 °C. 114 mg (0.94 mmol) of DMAP and 118 mg (0.94 mmol) of DIPC were added to the mixture. The reaction mixture was stirred overnight at ambient temperature. The solvent was removed *in vacuo* and the residue was recrystallized from 2-propanol to give 2.1 g (84%) of **18** as a white solid.

¹³C NMR (67.80 MHz, CDCl₃) δ 16.38, 19.46, 24.47, 24.75, 30.11, 30.36, 38.21, 43.66, 47.58, 58.12, 67.97-71.13 (PEG), 121.81, 131.80, 131.93, 135.68, 137.01, 148.89, 165.87, 168.55, 169.34, 171.03.

Example 12.

Compound 20: Conjugation of 18 to (L)-Asparaginase.

450 mg (0.083 mmol, 317 equiv.) of PEG linker, **18**, is added to 37.5 mg (416 μL, 0.00027 mmol) of native (L)-asparaginase, **19**, in 3 mL of sodium phosphate buffer (0.1 M, pH 7.8) with gentle stirring. The solution is stirred at 30 °C for 30 minutes. A GPC column (Zorbax GF-450) is used to monitor PEG conjugation: The PEG-Asp conjugate has a retention time of 8.5 min. At the end of the reaction (as evidenced by

the absence of native enzyme), the mixture was diluted with 12 mL of formulation buffer (0.05 M sodium phosphate, 0.85% sodium chloride, pH 7.3) and diafiltered with a Centriprep concentrator (Amicon) having a molecular weight cut-off 50,000 daltons to remove the unreacted PEG. Diafiltration is continued as needed at 4 °C until no more free PEG is detected by mixing equal amount of filtrate and 0.1% PMA (polymethacrylic acid in 0.1 M HCl).

The product, 20, is not stable in basic buffer solution for prolonged periods of time, therefore the solution is lyophilized and 20 is stored in the freezer (-20 °C). After 15 days of storage in this manner, GPC analysis indicates less than 0.8% decomposition. The specific activity of freshly prepared 20 is found to be 137 IU/mg (native asparaginase = 217 IU/mg). Protein modification of asparaginase with SS-PEG (a permanent linker) using a procedure corresponding to that described in the aforementioned U.S. Patent No. 4,179,337 gives a similar activity of 120 IU/mg. A TNBS assay is used to calculate the percentage modification of the protein, and the Biuret assay is used to check the protein concentration.

Example 13.

Kinetics of Hydrolysis of 20 in Rat Plasma and Buffer.

The rate of hydrolysis of 20 in rat plasma is measured using a GPC column (Zorbax GF-450) and is found to have a half life of <2 hours. The half life in phosphate buffer (pH 7.4) is determined as >>2 hours.

Example 14.

Compound 20A: A Protein Hybrid - Conjugation of 20 with SS-PEG (a permanent linker).

393 mg (0.073 mmol, 70 equiv.) of 18 is reacted with 150 mg (1.664 mL, 0.00106 mmol) of native (L)-asparaginase, 19, in 30 mL of sodium phosphate buffer (0.1 M, pH 7.8) as described in Example 14 at 30 °C for 15 minutes to provide a solution of 20, followed by the addition of 1.272 g (0.245 mmol, 230 equiv.) of SS-

PEG. The reaction solution is stirred for another 15 minutes. The pH of the reaction mixture is maintained at 7.8 with 0.5 M sodium hydroxide. The reaction mixture is diluted with 30 mL of sterile water and diafiltered using a Centriprep concentrator (Amicon) having a molecular weight cut-off of 50,000 daltons to remove any unreacted PEG. Diafiltration is continued as needed at 4 °C until no more free PEG is detected by mixing equal amount of filtrate and 0.1% PMA (polymethacrylic acid in 0.1 M HCl). A GPC column (Zorbax GF-450) is used to follow the course of the reaction. The final solution of product, 20A, is lyophilized and stored in the freezer.

Example 15.

Compound 20B: Demonstration of Selective Removal of Reversible PEG Linker from the Hybrid, 20A - Generation of a Permanently Modified Asparaginase, 20B.

100 mg of hybrid linker modified asparaginase, 20A, is dissolved in 30 mL of pH 7.8 phosphate buffer and stirred at 30 °C overnight. This solution is diluted with 30 mL of sterile water, and diafiltered with a Centriprep concentrator (Amicon) having a molecular weight cut off of 50,000 daltons to remove the free PEG which is formed by selective cleavage of the reversible PEG linker, 8 from the conjugate. The solution now contains only SS-PEG conjugated asparaginase, 20B. Thus the reversible linker is hydrolyzed, leaving only the relatively permanently bonded PEG attached to asparaginase.

Example 16.

Compound 22: *t*-Boc-Proline Ester of 1-*O*-*t*-Butyldimethylsilyl-3-(2'-hydroxy-4',6'-Dimethylphenyl)-3,3-dimethylpropanol, 21.

392 mg (3.11 mmol) of DIPC was added to a mixture of 0.5 g (1.55 mmol) of 1-*O*-*t*-Butyldimethylsilyl-3-(2'-hydroxy-4',6'-Dimethylphenyl)-3,3-dimethylpropanol, 21 (synthesized using the procedure of R. T. Borchardt, et al. Amine Prodrugs Which Utilize Hydroxy Amide Lactonization. II. A Potential Esterase-Sensitive Amide

Prodrug. *Pharmaceu. Res.* **1991**, *8*, 455), 568 mg (4.66 mmol) of DMAP, and 668 mg (3.11 mmol) of *t*-Boc-Proline-OH in 15 mL of anhydrous DCM at 0 °C. The mixture was stirred at room temperature overnight, filtered, and concentrated. The residue was purified by column chromatography on silica gel (ethyl acetate-hexane = 3 : 7, v/v) to give 700 mg (87%) of **22**.

¹H NMR (270 MHz, CDCl₃) δ 0.04, (s, 3H, CH₃-Si), 0.14 (s, 3H, CH₃-Si), 0.88 (s, 3H, CH₃-C), 0.92 (s, 3H, CH₃-C), 1.51 (s, 9H, Si(CH₃)₃), 1.55 (s, 9H, C(CH₃)₃), 2.00 (m, 2H, CH₂O), 2.07 (t, 2H, CH₂CH₂CH₂, *J* = 8.1 Hz), 2.25 (s, 3H, PhCH₃), 2.30 (m, 2H, CHCH₂) 2.55 (s, 3H, PhCH₃), 3.53 (t, 2H, CH₂N, *J* = 8.1 Hz), 4.57 (bs, 1H, COCHN), 6.65 (bs, 1H, PhH), 6.82 (s, 1H, PhH).

¹³C NMR (67.80 MHz, CDCl₃) δ 18.23, 20.06, 25.14, 25.70, 25.98, 28.49, 32.00, 32.06, 39.51, 46.24, 46.62, 59.74, 60.93, 80.08, 122.73, 132.14, 134.56, 135.87, 138.30, 150.68, 171.84.

Example 17.

Compound 23: *t*-Boc-Proline Ester of 3-(2'-hydroxy-4',6'-Dimethylphenyl)-3,3-dimethylpropanol.

A solution of 2.82 g (5.43 mmol) of **23** in 10 mL of tetrahydrofuran, 10 mL of H₂O and 30 mL of glacial acetic acid was stirred at room temperature for 1 hour. The solvent was removed *in vacuo* to give a crude product **23** as a colorless oil, which was carried to the next step without further purification.

¹H NMR (270 MHz, CDCl₃) δ 0.01, 0.77, 0.84, 1.38, 1.40, 1.42, 1.93, 1.97, 2.01, 2.13, 2.38, 2.43, 3.47, 4.48, 6.50, 6.70.

¹³C NMR (67.80 MHz, CDCl₃) δ -3.71, 19.95, 20.29, 23.19, 25.06, 25.20, 25.59, 28.44, 31.78, 32.04, 32.27, 33.21, 39.33, 42.96, 46.01, 46.53, 46.62, 59.62, 60.22, 64.95, 66.59, 80.44, 103.87, 122.59, 132.04, 132.27, 134.23, 135.79, 135.89, 138.14, 150.37, 150.55, 154.52, 171.86, 175.10.

Example 18.**Compound 24: Oxidation of *t*-Boc-Proline Ester of 3-(2'-hydroxy-4',6'-Dimethylphenyl)-3,3-dimethylpropanol, 23.**

A solution of 2.5 g (6.2 mmol) of **23** in 125 mL of anhydrous methylenechloride was added to a solution of 2.88 g (13.4 mmol) of pyridinium chlorochromate in 125 mL of anhydrous DCM. The mixture was stirred at room temperature for 1 hour followed by filtration through celite. The solvent was removed *in vacuo* and the residue was purified by silica gel column chromatography (ethyl acetate/hexane = 3:7, v/v). The product, aldehyde, was dissolved in 25 mL of acetone and added to a solution of 1 g (6.3 mmol) of KMnO₄ in 25 mL of acetone-H₂O (1:1, v/v). The mixture was stirred at room temperature overnight. 40 mL of H₂O was added and acetone was removed *in vacuo* before filtration through Celite. The aqueous solution was adjusted to pH = 3.0 with 1N HCl, followed by extraction with ethyl acetate (30 mL x 2). Solvent was removed under reduced pressure and hexane was added to precipitate **24** (550 mg, 21% from **23**).

¹H NMR (270 MHz, CDCl₃) δ 1.41, 1.46, 1.50, 1.52, 1.61, 1.97, 2.21, 2.30, 2.33, 2.54, 2.85, 3.56, 4.56, 6.57, 6.79.

¹³C NMR (67.80 MHz, CDCl₃) δ 20.09, 24.13, 25.17, 28.14, 28.56, 29.87, 31.40, 31.57, 39.12, 46.79, 47.40, 59.76, 80.68, 122.56, 132.43, 134.04, 136.27, 138.29, 150.21, 171.87.

Example 19.**Compound 25: Trifluoroacetic Acid Salt of Proline Ester of 3-(2'-hydroxy-4',6'-Dimethylphenyl)-3,3-dimethylpropionic Acid.**

0.55 g (1.31 mmol) of *t*-Boc-proline ester of 3-(2'-hydroxy-4',6'-dimethylphenyl)-3,3-dimethylpropionic acid, **24**, was stirred in 10 mL of TFA-CH₂Cl₂ (1:1, v/v) at room temperature for 1 h. Solvent was removed completely *in vacuo* to give **25** as a yellow foamy solid (0.436 g, 77%).

^1H NMR (270 MHz, CDCl_3) δ 1.53, 1.55, 2.03, 2.19, 2.48, 2.59, 2.76, 3.32, 4.61, 6.54, 6.85, 9.80.

^{13}C NMR (67.80 MHz, CDCl_3) δ 13.86, 23.73, 24.86, 28.27, 31.70, 31.92, 38.99, 46.43, 47.76, 60.33, 122.25, 133.08, 133.74, 136.65, 138.89, 149.30, 168.35.

5 **Example 20.**

Compound 26: Coupling of 25 with PEG (40 kDa) Dithiazolidine thione, 10.

52.6 mg (0.41 mmol) of DIPEA was added to the solution of 65 mg (0.15 mmol) of **25** and 1 g (0.025 mmol) of **10** in 15 mL of anhydrous DCM. The mixture was stirred at room temperature overnight. The solvent was removed *in vacuo* and the residue was recrystallized from 2-propanol to give 0.85 g (85%) of **26** as a white solid.

10 ^{13}C NMR (67.80 MHz, CDCl_3) δ 19.61, 24.39, 27.34, 30.72, 38.11, 45.86, 59.13, 68.40-71.45 (PEG), 122.18, 131.78, 133.40, 135.04, 137.18, 149.36, 168.65, 170.67, 171.18.

Example 21.

15 **Compound 27: Coupling of 26 with 5.**

13.2 mg (0.07 mmol) of EDC was added to the mixture of 0.35 g (0.01 mmol) of **26**, 29 mg (0.05 mmol) of **5**, 13.9 mg (0.14 mmol) of NMM, and 6.97 mg (0.05 mmol) of HOBT in 20 mL of anhydrous DCM at 0 °C. The reaction mixture was stirred at room temperature overnight and filtered. The filtrate was concentrated *in vacuo* and the residue was recrystallized from 2-propanol (50 mL) to give 0.35 g (84%) of **27**. UV assay showed 2.0 equivalents (100% of coupling yield) of **5**.

20

Example 22.

Compound 29.

Compound **28** was prepared using the procedure provided in Example 16. Thus 500 mg (1.55 mmol) of **21**, 587.6 mg (3.11 mmol) of *t*-Boc- β -alanine-OH, 474 μL (3.11 mmol) of DIPC, and 568 mg (4.66 mmol) of DMAP in 15 mL of anhydrous DCM were reacted to give 580 mg (76%) of **28**.

25

^1H NMR (270 MHz, CDCl_3) δ 0.00 (s, 6H, 2 x $\text{CH}_3\text{-Si}$), 0.87 (s, 6H, 2 x $\text{CH}_3\text{-C}$), 1.48 (s, 18H, $\text{C}(\text{CH}_3)_3$ and $\text{Si}(\text{CH}_3)_3$), 2.05 (t, 2H, CH_2C , $J = 5.4$ Hz), 2.26 (s, 3H, PhCH_3), 2.54 (s, 3H, PhCH_3), 2.79 (t, 2H, $\text{CH}_2\text{C(=O)}$, $J = 5.4$ Hz), 3.50 (t, 4H, NHCH_2 and CH_2O , $J = 8.1$ Hz), 5.01 (bs, 1H, NH), 6.58 (s, 1H, PhH), 6.84 (s, 1H, PhH).

^{13}C NMR (67.80 MHz, CDCl_3) δ 18.18, 20.11, 25.20, 25.88, 28.36, 31.78, 35.40, 36.00, 38.99, 45.94, 60.69, 79.35, 122.91, 132.38, 133.99, 135.92, 138.40, 149.46, 155.79, 171.73.

Compound **29** is prepared using the same procedure as described for the conversion of **22** into **27** (Examples 17-21).

Example 23.

Compound 31.

Compound **30** was prepared using the procedure given in Example 16.

Compound **21**, 150 mg (0.47 mmol), 189.4 mg (0.93 mmol) of *t*-Boc- α -aminoisobutyric acid, 117.4 mg (0.93 mmol) of DIPC, and 113.7 mg (0.93 mmol) of DMAP in 25 mL of anhydrous DCM were reacted and after purification, 76 mg (32%) of **30** was obtained.

^1H NMR (270 MHz, CDCl_3) δ 0.00 (s, 6H, 2 x $\text{CH}_3\text{-Si}$), 0.87 (s, 6H, 2 x $\text{CH}_3\text{-C}$), 1.48 (s, 18H, $\text{C}(\text{CH}_3)_3$ and $\text{Si}(\text{CH}_3)_3$), 1.70 (s, 6H, $\text{C}(\text{CH}_3)_2$), 2.06 (t, 2H, CH_2C , $J = 8.1$ Hz), 2.19 (s, 3H, PhCH_3), 2.54 (s, 3H, PhCH_3), 3.50 (t, 2H, CH_2O , $J = 8.1$ Hz), 5.19 (bs, 1H, NH), 6.70 (s, 1H, PhH), 6.81 (s, 1H, PhH).

^{13}C NMR (67.80 MHz, CDCl_3) δ 18.16, 50.13, 25.17, 25.33, 25.90, 28.33, 31.92, 32.04, 39.28, 45.08, 45.83, 56.44, 60.80, 61.61, 79.96, 116.65, 122.70, 126.74, 132.17, 134.12, 136.07, 138.04, 151.07, 154.70, 174.24.

Compound **30** was subjected to the procedures of Examples 17-21 to obtain compound **31**.

Example 24.**Compound 33: Coupling of 8 with Ara-C, 32.**

A solution of 300 mg (0.056 mmol) of **8**, 68.3 mg (0.28 mmol) of Ara-C, **32**, and 65 mg (0.34 mmol) of EDC in 10 mL of anhydrous pyridine was stirred at room temperature overnight. Ethyl ether was added to the reaction solution to precipitate the crude product which was recrystallized from 2-propanol to give 180 mg (53%) of **33**.

^{13}C NMR (67.80 MHz, CDCl_3) δ 16.07, 19.85, 25.35, 31.56, 32.11, 39.00, 48.39, 49.40, 58.61, 61.51, 68.53-72.18 (PEG), 74.70, 85.43, 87.72, 95.36, 122.00, 132.30, 132.34, 133.32, 135.74, 138.27, 145.62, 149.35, 155.46, 161.74, 171.37, 172.04, 172.20.

Example 25.**Compound 34:**

Compound **34** can be prepared from **11** coupled with **32** or from **1** coupled with **32** followed by deprotection and coupling with **10** as in Examples 26-28.

Coupling of 11 with 32: A solution of 1 g (0.025 mmol) of **11**, 120 mg (0.50 mmol) of **32** and 57 mg (0.30 mmol) of EDC in 10 mL of anhydrous pyridine was stirred at room temperature for 3 days. The solvent was removed *in vacuo* and the residue was recrystallized from IPA to give 640 mg (64%) of **34**. UV assay showed 1.67 equivalents (84% of coupling yield) of **32**.

^{13}C NMR (67.80 MHz, CDCl_3) δ 14.87, 18.58, 24.54, 31.30, 31.36, 38.66, 47.68, 49.09, 61.12, 67.54-71.05 (PEG), 74.76, 85.19, 86.54, 94.69, 121.25, 131.85, 133.08, 134.73, 137.35, 144.94, 148.58, 154.39, 160.88, 170.96, 171.26, 172.38.

Example 26.**Compound 35: Coupling of 1 with 32.**

A mixture of 700 mg (1.78 mmol) of **1**, 1.73 g (7.12 mmol) of **32**, 0.96 g (7.12 mmol) of HOBT, and 2.73 g (14.25 mmol) of EDC in 50 mL of anhydrous pyridine

was stirred at room temperature for 2 h and followed by stirring at 40 °C overnight. The solvent was removed and 50 mL of DCM was used to dissolve the mixture followed by washing with water (3 x 30 mL) and 0.1 N HCl (2 x 30 mL). The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed *in vacuo* to give the crude product which was purified by silica gel column chromatography (5 to 10% methanol in DCM) to give 638.8 mg (52%) of **35** as a white solid.

¹H NMR (270 MHz, CDCl₃) δ 1.42, 1.55, 2.17, 2.26, 2.46, 2.79, 3.84, 3.91, 4.14, 4.33, 4.53, 5.49, 6.07, 6.17, 6.52, 6.76, 7.31, 7.67, 8.16, 8.62.

¹³C NMR (67.80 MHz, CDCl₃) δ 17.77, 20.11, 25.36, 28.32, 31.51, 31.96, 39.57, 50.18, 50.45, 61.88, 74.50, 80.15, 85.90, 88.58, 96.25, 122.51, 132.82, 133.34, 136.73, 138.22, 146.57, 149.90, 155.65, 155.96, 162.08, 171.89, 174.06.

Example 27.

Compound 36: Deprotection of 35.

Compound **35** (638.8 mg, 1.03 mmol) was stirred in 6 mL of anhydrous DCM 6 mL and 4 mL of TFA at room temperature for 2 h. Ethyl ether was added to the solution to precipitate the crude product which was filtered and washed with ether to give **36** as a white solid (534.5 mg, 82%).

¹H NMR (270 MHz, DMSO-*d*₆) δ 1.52 (s, 3H, (CH₃)₂CH) 1.55 (s, 3H, (CH₃)₂CH), 1.62 (d, 1 H, *J* = 8.1 Hz, (CH₃)₂CH), 2.22 (s, 3H, CH₃Ar), 2.57 (s, 3H, CH₃Ar), 2.97 (s, 2H, CH₂C(=O)), 3.41-4.27 (m, 5 H, Ara-C's H-2'-H5'), 6.09 (d, 1H, *J* = 5.4 Hz, Ara-C's H-1'), 6.67 (s, 1H, Ar-H), 6.90 (s, 1H, Ar-H), 7.12 (d, *J* = 5.4 Hz, H-6), 8.05 (d, *J* = 8.1 Hz, H-5), 8.67 (bs, 1H, TFA).

¹³C NMR (67.80 MHz, DMSO-*d*₆) δ 15.45, 19.67, 24.97, 31.05, 31.23, 38.56, 40.41, 48.53, 49.02, 61.02, 64.94, 74.64, 76.14, 85.74, 86.95, 94.32, 122.32, 132.41, 134.08, 135.67, 138.09, 146.71, 149.20, 154.50, 158.21, 158.72, 162.02, 169.68, 171.87.

Example 28.**Compound 34 by Coupling of 10 with 36.**

A solution of 778 mg (0.019 mmol) of **10**, 40 mg (0.077 mmol) of **36**, and 20 mg (0.15 mmol) of DIPEA in 10 mL of anhydrous DCM was stirred at room temperature overnight. The solvent was removed *in vacuo* and the residue was recrystallized from 2-propanol to give 650 mg (84 % yield) of **34** as a white solid. The NMR data were correspondent with the product from Example 25.

Example 29.**Compound 38: Coupling of 1 with Gemcitabine, 37.**

A mixture of 359 mg (0.91 mmol) of **1**, 960 mg (3.65 mmol) of gemcitabine, 492 mg (3.65 mmol) of HOBT, 737 mg (7.29 mmol) of NMM, and 390 mg (2.04 mmol) of EDC in 25 mL of anhydrous pyridine was stirred at room temperature for 3 days. The mixture was concentrated *in vacuo* and the residue was purified by column chromatography (ethyl acetate) to give 280 mg (48 %) of **38**.

¹H NMR (270 MHz, CDCl₃) δ 1.26 (s, 3H), 1.28 (s, 3H), 1.41 (s, 3H), 1.52 (s, 3H), 1.55 (s, 3H), 1.63 (bs, 3H), 2.19 (s, 3H), 2.53 (s, 3H), 2.91 (dd, 2H, *J* = 29.7, 16.2 Hz), 3.96 (m, 2H), 4.50 (bs, 1H), 5.41 (bs, 1H), 6.21 (bs, 1H), 6.50 (s, 1H), 6.80 (s, 1H), 7.35 (d, 1H, *J* = 5.4 Hz), 8.02 (d, 1H, *J* = 8.1 Hz), 9.37 (bs, 1H).

¹³C NMR (67.80 MHz, CDCl₃) δ 17.40, 20.16, 25.54, 26.89, 28.28, 29.68, 31.36, 31.93, 39.29, 50.05, 50.53, 59.57, 68.96, 80.68, 81.50, 97.50, 122.46, 132.92, 133.49, 136.66, 138.25, 144.97, 149.64, 155.75, 162.89, 172.04, 173.48.

Example 30.**Compound 39: Deprotection of 38.**

Compound **38** (280 mg, 0.44 mmol) was stirred in 5 mL of TFA and 5 mL of anhydrous DCM at room temperature for 1 h. The solvent was removed *in vacuo* and ethyl ether was added to precipitate the product which was collected by filtration and washed with ethyl ether to give 250 mg (87%) of **39**.

¹H NMR (270 MHz, DMSO-*d*₆) δ 1.48 (d, 6H, *J* = 8.1 Hz) 1.58 (d, 6H, *J* = 5.4 Hz), 2.18 (s, 5H), 2.95 (s, 3H), 3.61-3.66 (m, 1H), 3.77-3.90 (m, 2H), 4.14 (m, 1H), 4.45 (m, 1H), 4.72 (m, 1H), 5.23 (bs, 1H), 6.16 (t, 1H, *J* = 8.1 Hz), 6.34 (bs, 1H), 6.60 (s, 1H), 6.88 (s, 1H), 7.13 (d, 1H, *J* = 8.1 Hz), 8.18 (d, 1H, *J* = 8.1 Hz), 8.47 (bs, 3H).

¹³C NMR (67.80 MHz, DMSO-*d*₆) δ 15.38, 19.63, 24.92, 30.99, 31.07, 31.15, 48.47, 49.12, 58.73, 68.35, 81.07, 95.81, 122.19, 122.91, 132.41, 133.95, 135.65, 138.09, 144.75, 149.07, 154.14, 162.69, 169.65, 172.05.

Example 31.

Compound 40: Coupling of 39 with 10.

A solution of 778 mg (0.019 mmol) of **10**, 50 mg (0.077 mmol) of **39**, and 20 mg (0.15 mmol) of DIPEA in 10 mL of anhydrous DCM is stirred at room temperature overnight. The solvent is removed *in vacuo* and the residue is recrystallized from 2-propanol to give **40**.

Example 32.

Compound 42: Coupling of 11 with Melphalan, 41.

A mixture of 1.0 g (0.025 mmol) of **11**, 13.7 mg (0.10 mmol) of *p*-nitrophenol, 12.4 mg (0.10 mmol) of DIPC, and 12.0 mg (0.10 mmol) of DMAP in 10 mL of anhydrous DCM was stirred 0 °C to room temperature overnight. The solution was concentrated and ethyl ether was used to precipitate a solid, which was used for next step without further purification. The *p*-nitrophenol ester of **11** was reacted with 45 mg (0.15 mmol) of melphalan in the presence of 51 mg (0.40 mmol) of DIPEA in 10 mL of anhydrous *N,N*-dimethylformamide at room temperature overnight. Ethyl ether was added to precipitate the crude product which was recrystallized from 2-propanol to give 700 mg (70 %) of product.

¹³C NMR (67.80 MHz, CDCl₃) δ 16.35, 16.70, 19.59, 20.64, 21.31, 24.67, 25.12, 31.04, 31.15, 36.02, 38.47, 39.12, 40.01, 47.18, 47.40, 48.35, 52.84, 60.62,

68.08-71.63 (PEG), 111.22, 125.57, 130.44, 131.67, 131.72, 133.81, 135.66, 135.90, 137.36, 143.80, 144.17, 149.02, 154.62, 168.34, 169.26, 168.25, 171.8, 171.52.

Example 33.

Compound 46.

5 Compound 44 was prepared from 2,5-dimethylphenol, 43, using a reported procedures (L. A. Cohen, et al., *J. Am. Chem. Soc.*, **1972**, *94*, 9158.).

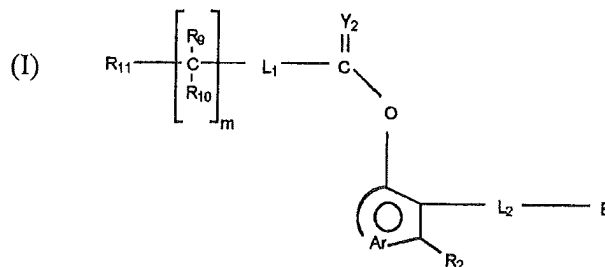
 Compound 44 is converted to compound 45 by the method used for the preparation of compound 1 as in the reference. Compound 45 is subjected to the procedures of Examples 29-31 to obtain compound 46.

10 The various publications, patents, patent applications and published applications mentioned in this application are hereby incorporated by reference herein.

 While there have been described what are presently believed to be the preferred embodiments of the invention, those skilled in the art will realize that changes and modifications may be made without departing from the spirit of the invention. It is
15 intended to claim all such changes and modifications as fall within the true scope of the invention.

WE CLAIM:

1. A compound comprising the formula:



wherein:

B is H, OH, OSiR_{13} , a residue of an amine-containing target moiety or a residue of a hydroxyl-containing moiety;

L_1 and L_2 are bifunctional linking moieties;

Y_2 is O or S;

R_2 is selected from the group consisting of C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy, and C_{1-6} heteroalkoxy;

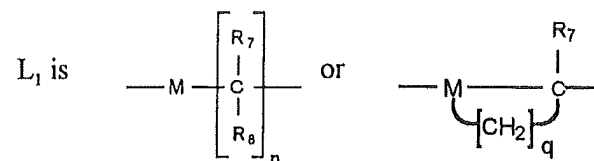
R_9 , R_{10} , and R_{13} are independently one of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy, and C_{1-6} heteroalkoxy;

Ar is a moiety which when included in Formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group;

(m) is zero or one; and

R_{11} is a polymer residue.

2. The compound of claim 1, wherein



wherein:

M is X or Q; wherein

X is an electron withdrawing group

Q is a moiety containing a free electron pair positioned three to six atoms from C(=Y₂);

(n) is zero or a positive integer;

(q) is three or four; and

R₇ and R₈ are independently selected from the group which defines R₉.

3. The compound of claim 1, wherein L₂ is a $\text{---} \begin{array}{c} R_1 \\ | \\ C \\ | \\ R_4 \end{array} \text{---} [CH_2]_p \text{---} G \text{---}$
 wherein:

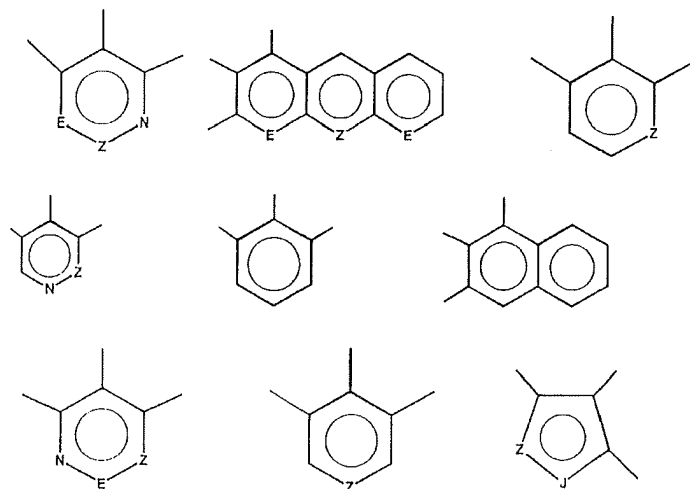
G is $\begin{array}{c} Y_1 \\ || \\ -C- \end{array}$ or CH₂; wherein

Y₁ is O or S;

(p) is zero, one or two; and

R₁ and R₄ are independently selected from the group which defines R₂.

4. The compound of claim 1, wherein Ar is selected from the group consisting of:

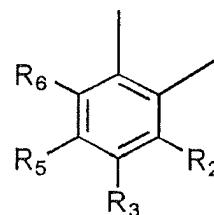


wherein J is O, S or NR₁₄;

E and Z are independently CR₁₄ or NR₁₄; and

R₁₄ is independently selected from the group which defines R₉.

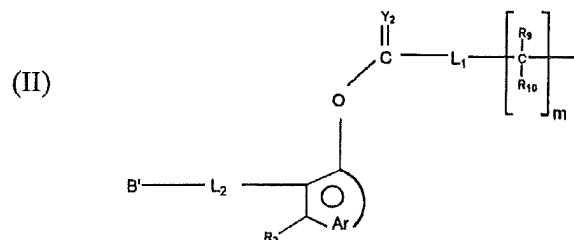
5. The compound of claim 1, wherein Ar is



wherein R₃, R₅, and R₆ are independently selected from the group which defines R₉ or a cyano, nitro, carboxyl, acyl, substituted acyl, carboxyalkyl.

- 6 The compound of claim 5, wherein R₂ and R₅ are independently selected from the group consisting of C₁₋₆ alkyls.
- 7 The compound of claim 6, wherein R₂ and R₅ are methyl.
- 8 The compound of claim 5, wherein R₃ and R₆ are hydrogen.
- 9 The compound of claim 1, wherein R₁₁ further comprises a capping group A.

10. The compound of claim 9, wherein A is selected from the group consisting of hydrogen, CO₂H, C₁₋₆ alkyl moieties, dialkyl acyl urea alkyls and



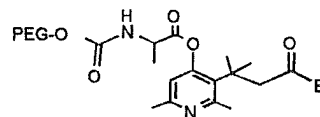
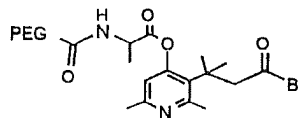
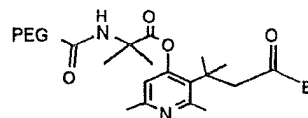
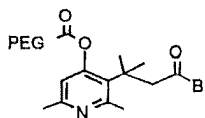
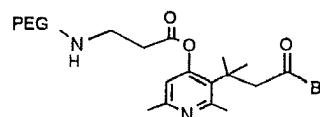
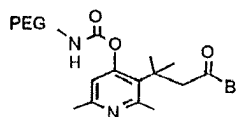
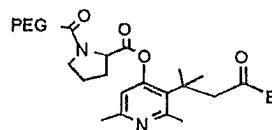
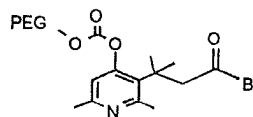
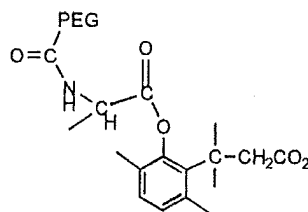
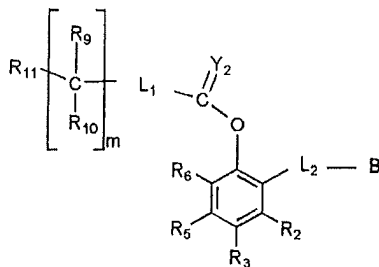
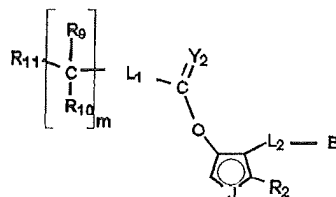
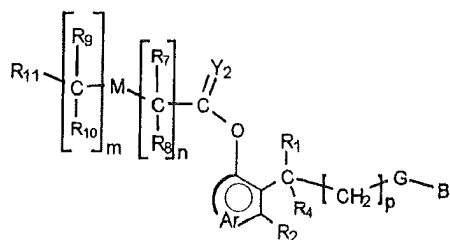
wherein B' is the same as B or another member of the group defined by B.

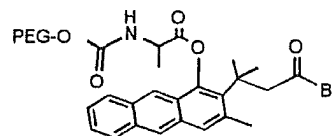
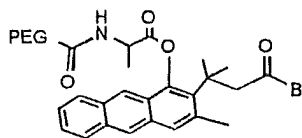
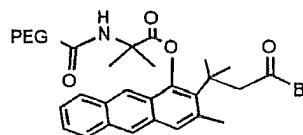
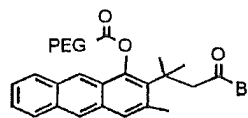
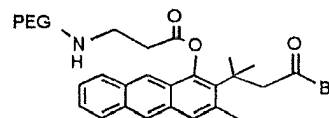
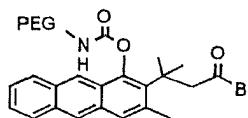
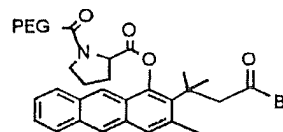
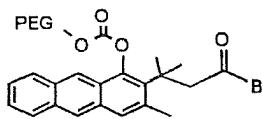
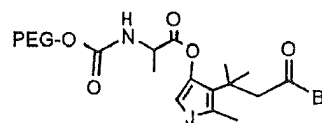
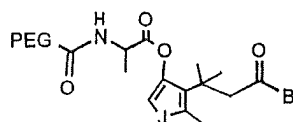
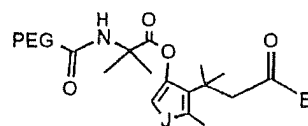
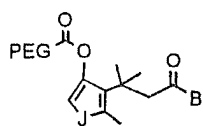
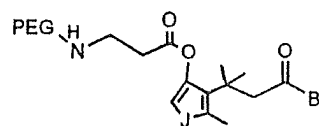
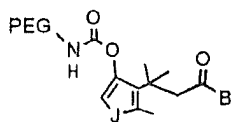
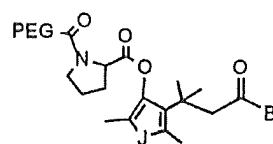
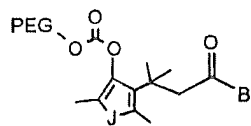
11. The compound of claim 3, wherein R₁ and R₄ are independently selected from the group consisting of CH₃ and CH₂CH₃.
12. The compound of claim 1, wherein X is selected from the group consisting of O, NR₁₂, S, SO and SO₂, where R₁₂ is selected from the group consisting of hydrogen, C₁₋₆ alkyls, C₃₋₁₂ branched alkyls, C₃₋₈ cycloalkyls, C₁₋₆ substituted alkyls, C₃₋₈ substituted cycloalkyls, aryls, substituted aryls, aralkyls, C₁₋₆ heteroalkyls and substituted C₁₋₆ heteroalkyls.
13. The compound of claim 12, wherein X is selected from the group consisting of O and NR₁₂.
14. The compound of claim 2, wherein Q is selected from the group consisting of C₂₋₄ alkyls, cycloalkyls, aryls, aralkyl groups substituted with a member of the group consisting of NH, O, S, -CH₂-C(O)-NH-, and ortho-substituted phenyls.
15. The compound of claim 2, wherein (*n*) is an integer from 1 to about 12.
16. The compound of claim 15, wherein (*n*) is 1 or 2.
17. The compound of claim 1, wherein (*m*) is 0.
18. The compound of claim 3, wherein (*p*) is one.
19. The compound of claim 2, wherein Y₁₋₂ are both O.
20. The compound of claim 1, wherein R₁₁ comprises a polyalkylene oxide.

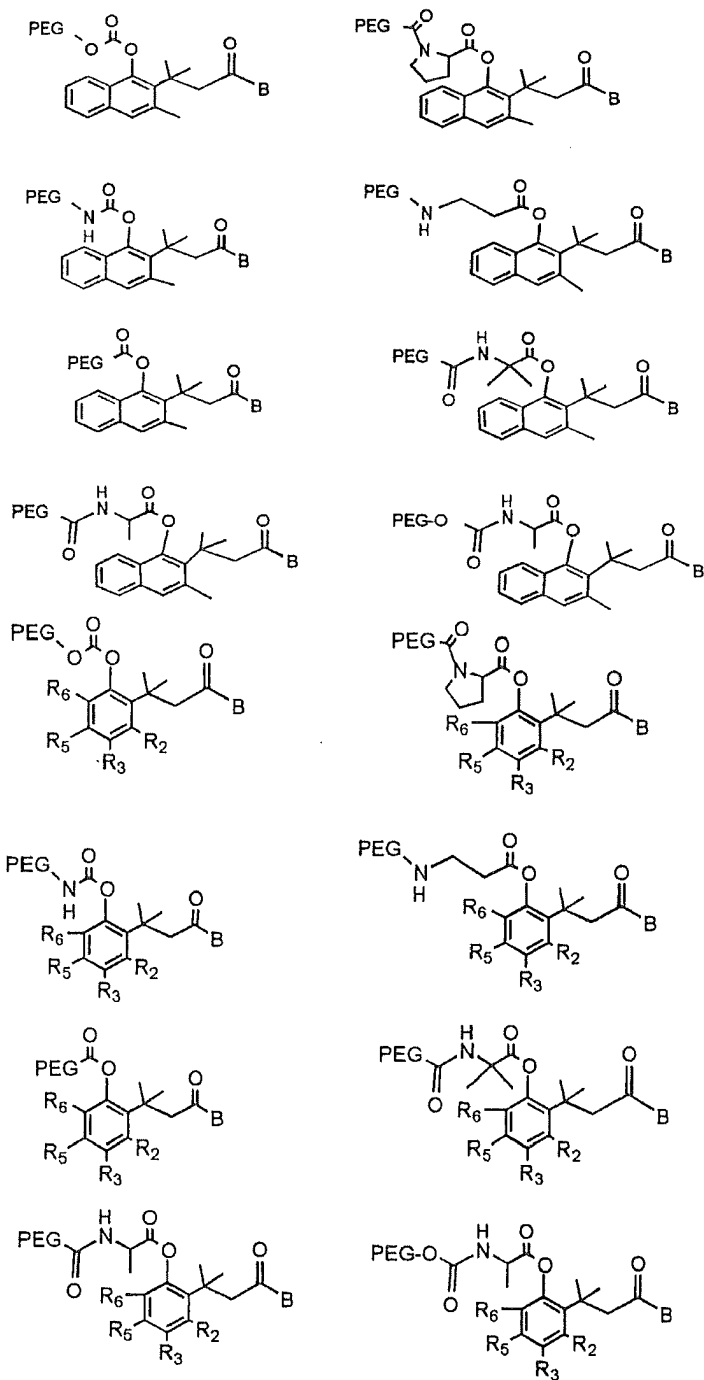
21. The compound of claim 20, wherein said polyalkylene oxide comprises polyethylene glycol.
22. The compound of claim 1, wherein said polymer residue has a molecular weight of from about 2,000 to about 100,000.
23. The compound of claim 22, wherein said polymer residue has a molecular weight of from about 5,000 to about 40,000.
24. The compound of claim 1, wherein R_{11} is selected from the group consisting of $-C(=Y)-(CH_2)_a-O-(CH_2CH_2O)_x-A$, $-C(=Y)-Y-(CH_2)_a-O-(CH_2CH_2O)_x-A$ and $-C(=Y)-NR_{12}-(CH_2)_a-O-(CH_2CH_2O)_x-A$, wherein (a) is zero or a positive integer; Y is O or S; A is a capping group; and (x) represents the degree of polymerization.
25. The compound of claim 1, wherein B is a leaving group selected from the group consisting of N-hydroxybenzotriazolyl, halogen, N-hydroxyphthalimidyl, *p*-nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, thiazolidinyl thione, and an acid activating group.
26. The compound of claim 1 wherein B is a residue of a member of the group consisting of anthracyclines, daunorubicin, doxorubicin, *p*-hydroxyaniline mustard, Ara-C, cytosine arabinoside and gemcitabine.
27. The compound of claim 1, wherein B is a residue of an enzyme, protein, peptide or an amine containing compound selected from the group consisting of cardiovascular agents, anti-neoplastics, anti-infectives, anti-fungals, anti-anxiety agents, gastrointestinal agents, central nervous system-activating agents, analgesics, fertility agents, contraceptive agents, anti-inflammatory agents, steroidal agents, anti-urecemic agents, vasodilating agents, and vasoconstricting agents.

28. The compound of claim 1, wherein B includes a second polymeric transport system.

29. A compound of claim 1, selected from the group consisting of:

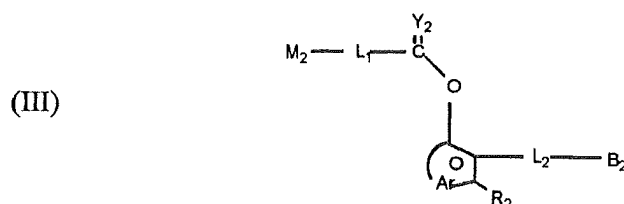






30. A method for preparing a prodrug transport form, comprising:

a. providing an intermediate compound (III)



wherein M₂ is a cleavable or reversible protecting group;

L₁ and L₂ are bifunctional linking moieties;

B₂ is a leaving group;

Y₂ is O or S; and

Ar is a moiety which when included in Formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group;

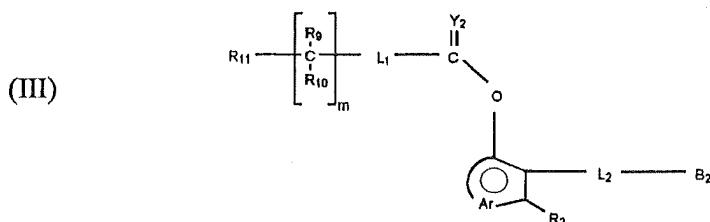
b. treating the intermediate compound (III) with an acid or catalytic hydrogenation to remove the protecting group; and

c. reacting the unprotected intermediate compound (III) with a moiety capable of reacting with a L₁ such as an activated polymer.

31. The method of claim 30, further comprising the step of:

d. reacting the resultant compound of step c) with an amine-containing or hydroxyl-containing compound to form a conjugate.

32. A method for preparing a prodrug transport form, comprising:
- a. providing an intermediate compound (III)



wherein M_2 is a cleavable or reversible protecting group;

L_1 and L_2 are bifunctional linking moieties;

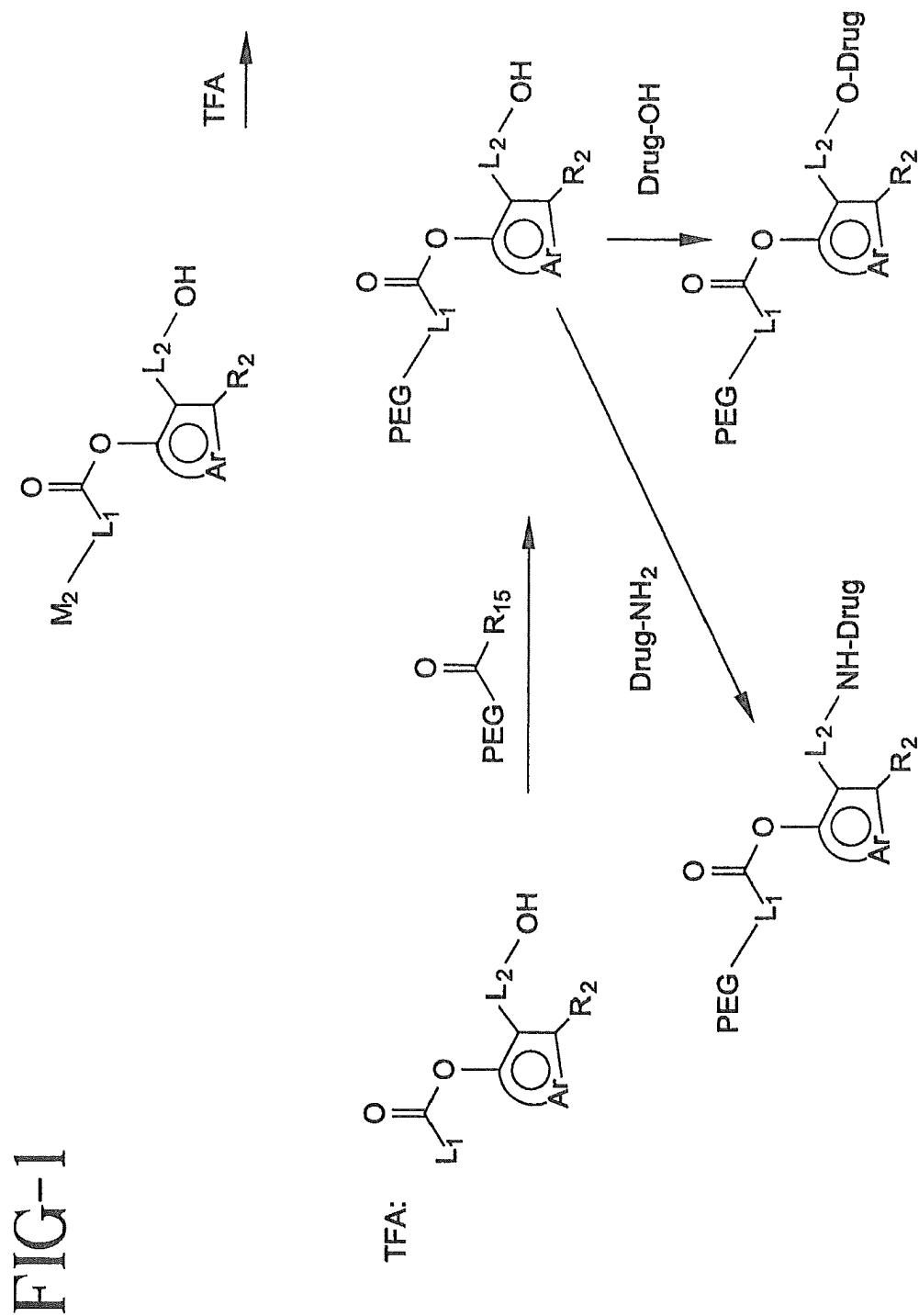
B_2 is a leaving group;

Y_2 is O or S; and

Ar is a moiety which when included in Formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group;

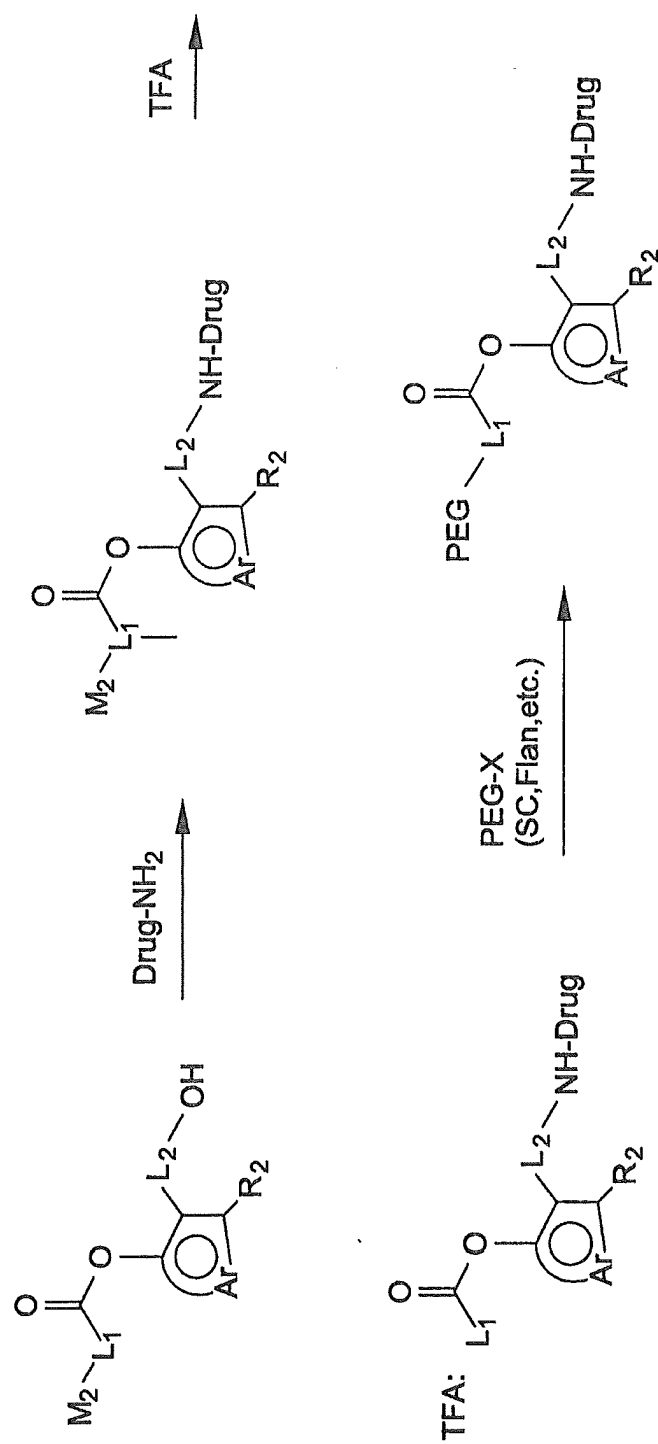
- b. coupling the intermediate compound (III) to an amine-containing or a hydroxyl-containing compound to form a second intermediate;
 - c. deprotecting the second intermediate with an acid; and
 - d. reacting the unprotected second intermediate with an activated polymer.
- 33 The method of claim 32, further comprising reacting the compound of formula (I) with an activated polymer to form a hybrid transport system.
34. A method of treating a mammal with prodrugs, comprising:
- administering to a mammal in need of such treatment an effective amount of a composition of claim 1 wherein B is a residue of an amine-containing or hydroxyl-containing therapeutic moiety.

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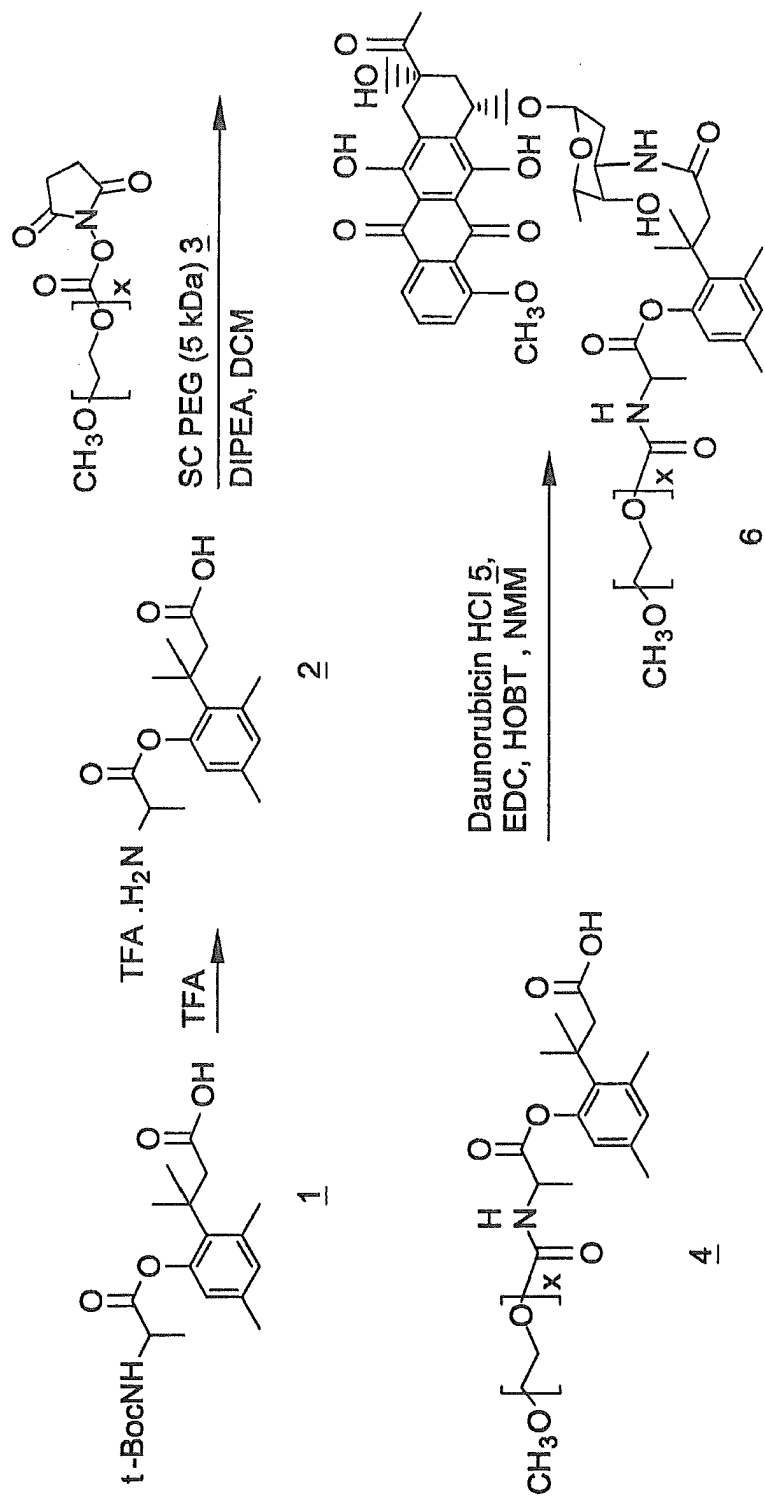
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FIG-2



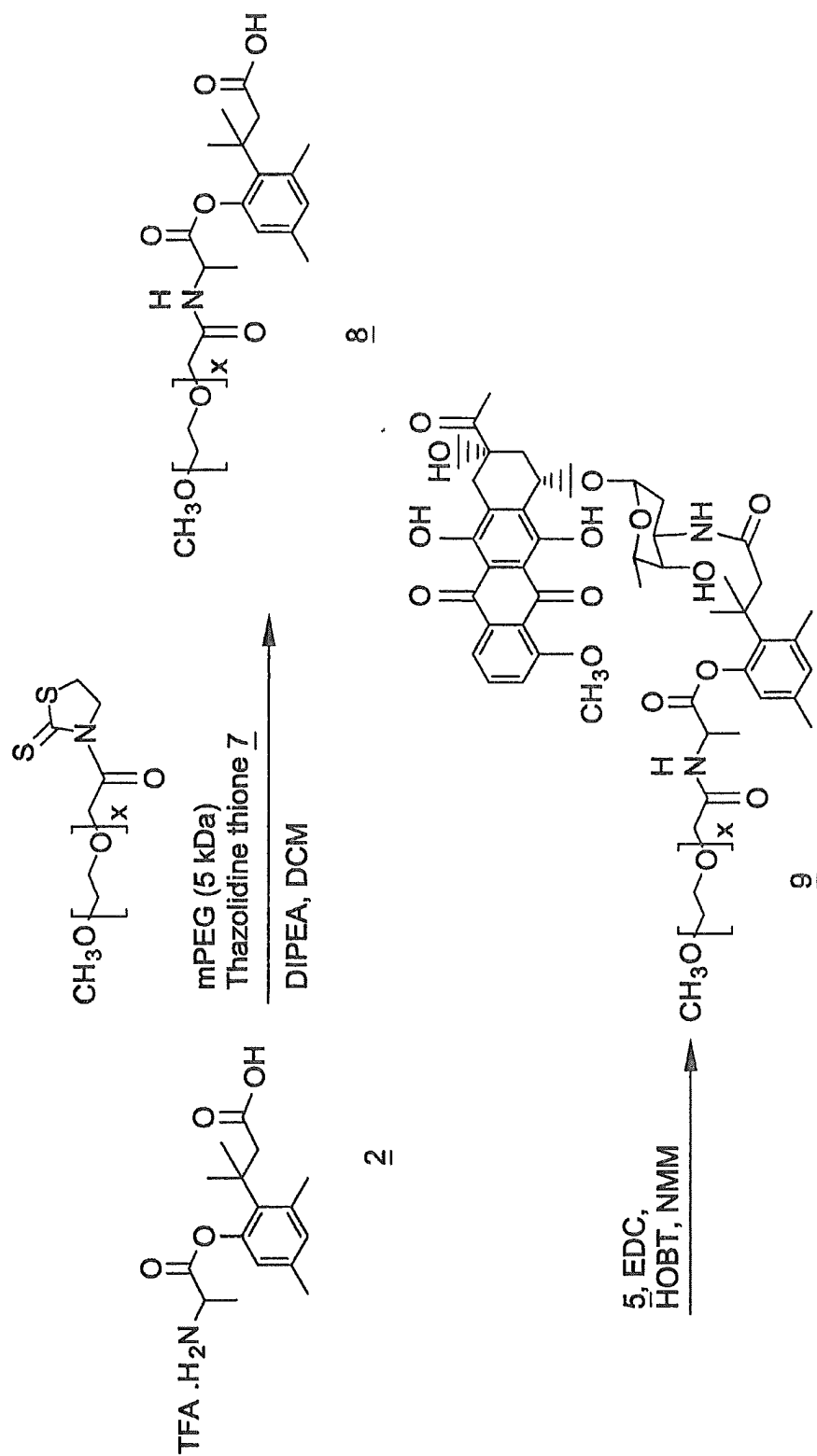
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FIG-3

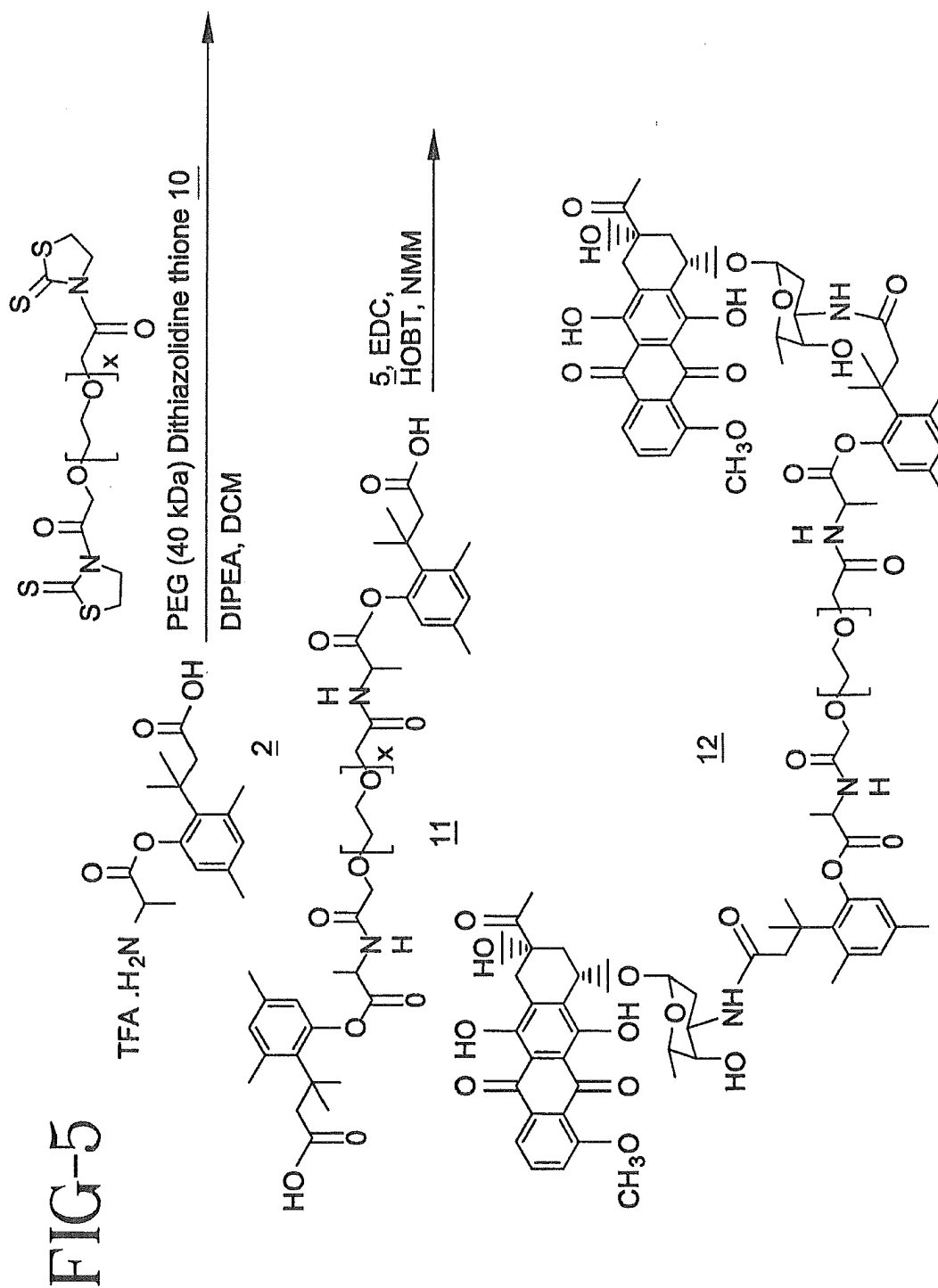


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FIG-4

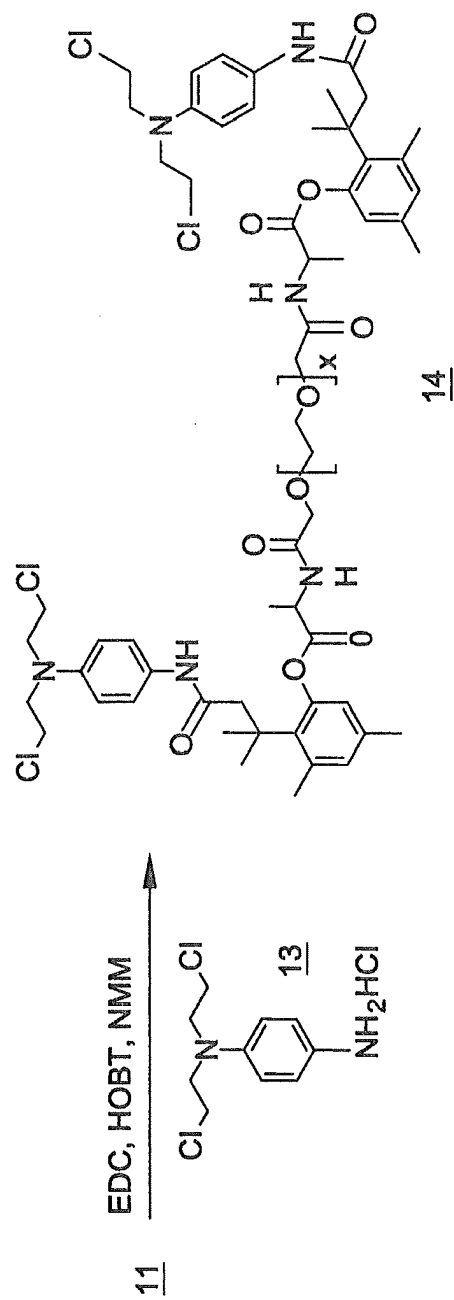


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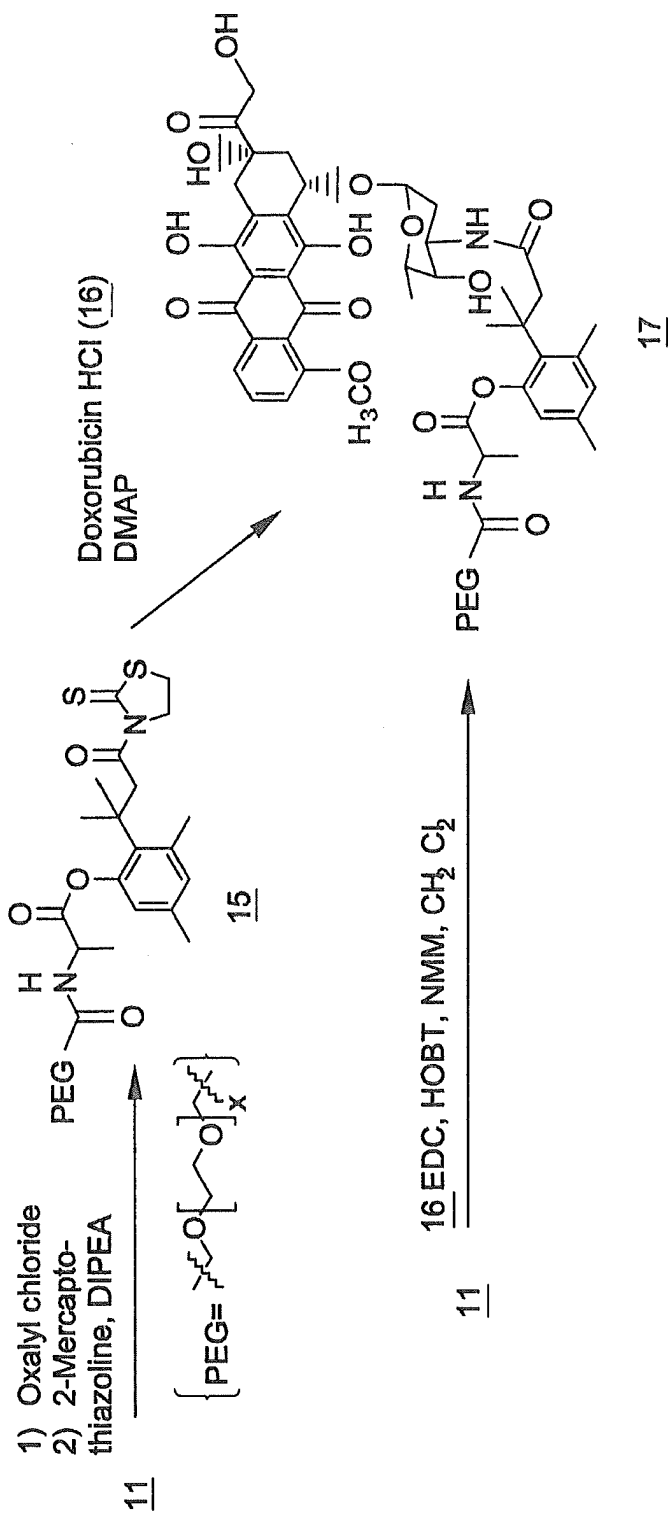
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FIG-6

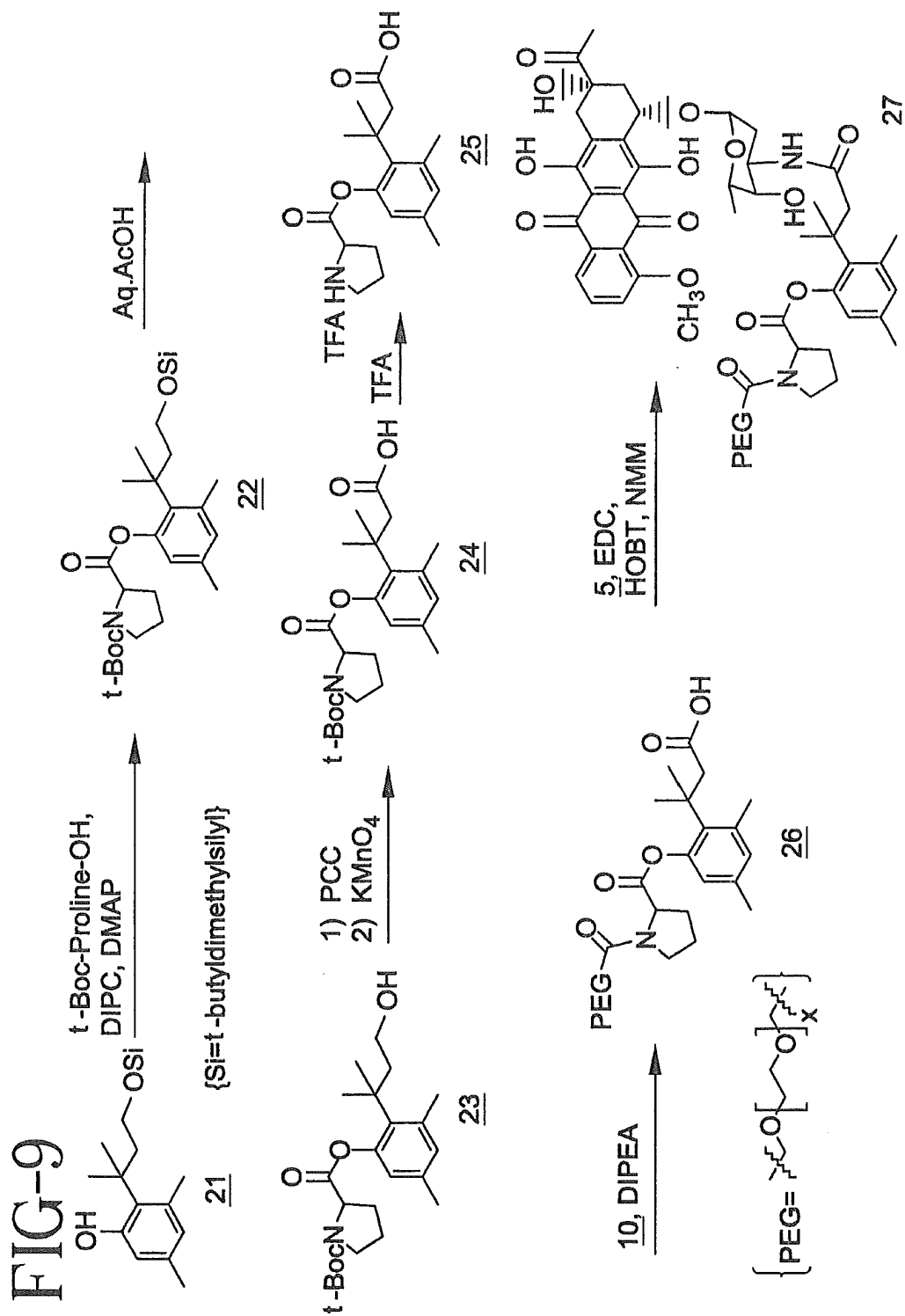


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FIG-7

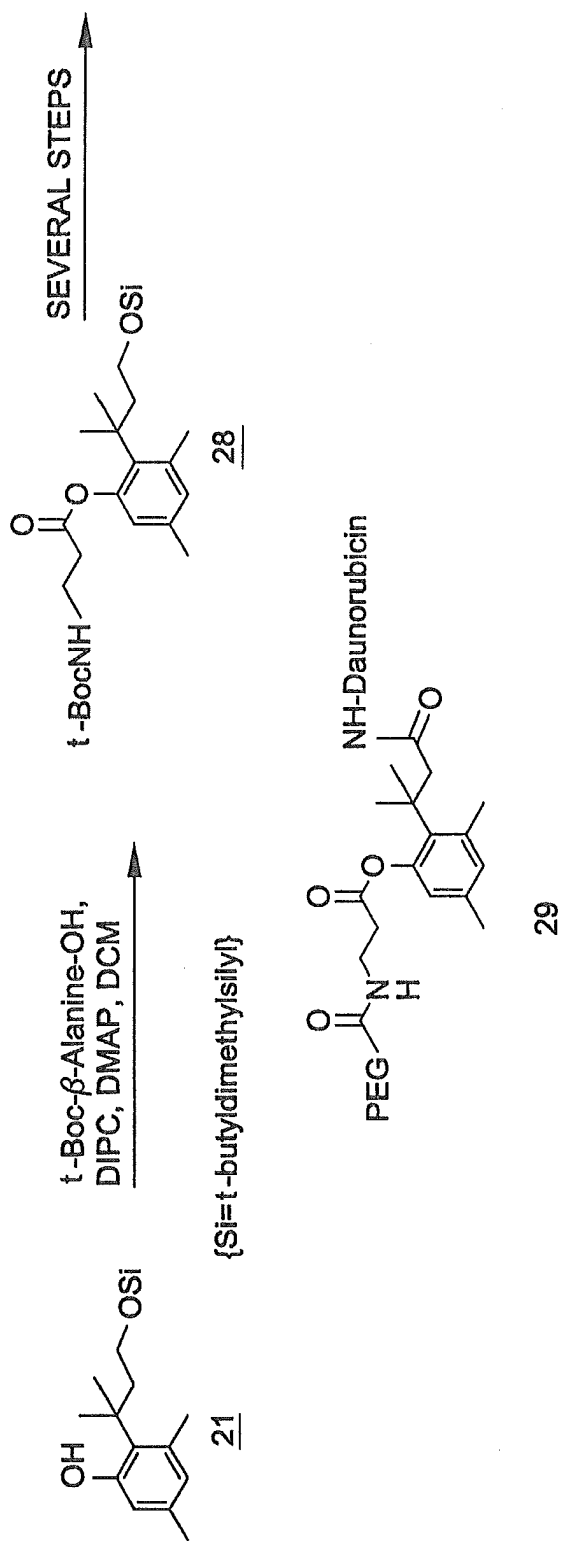


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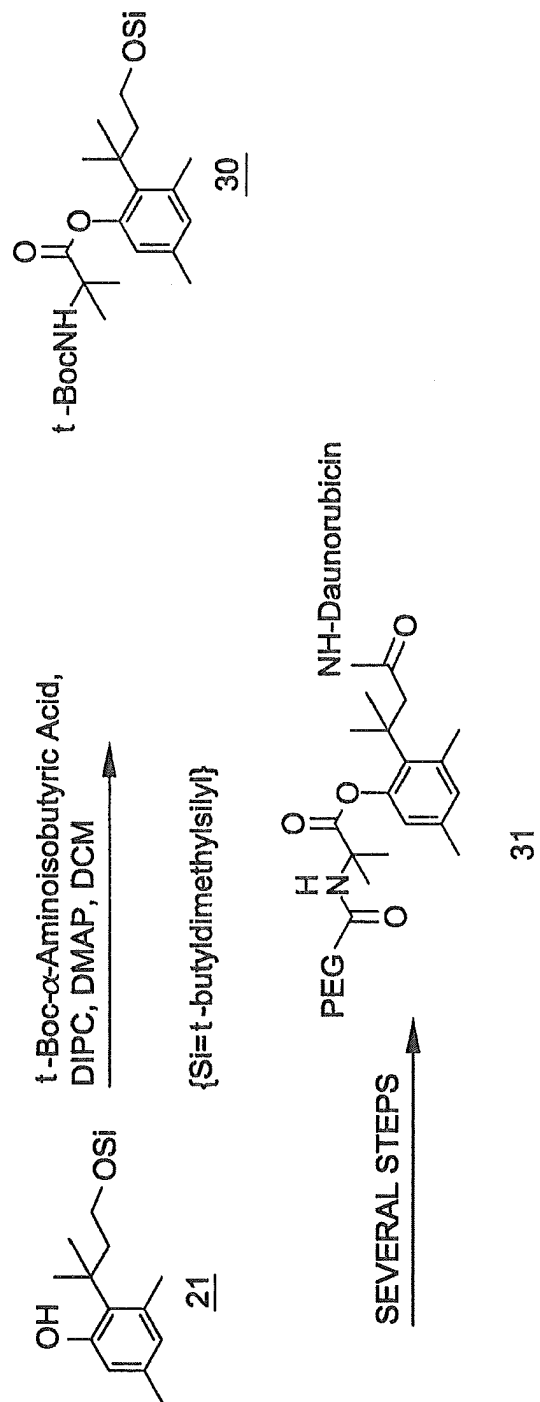
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FIG-10



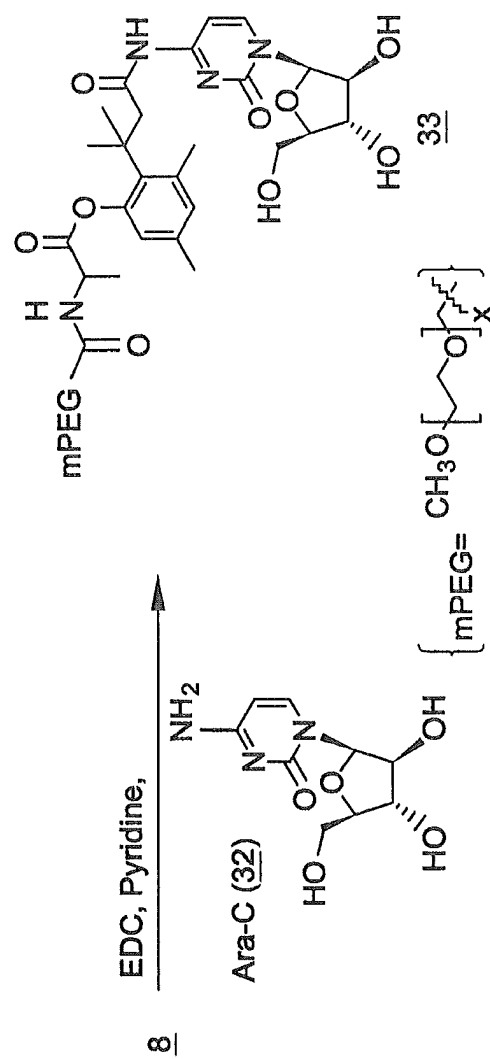
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FIG-11



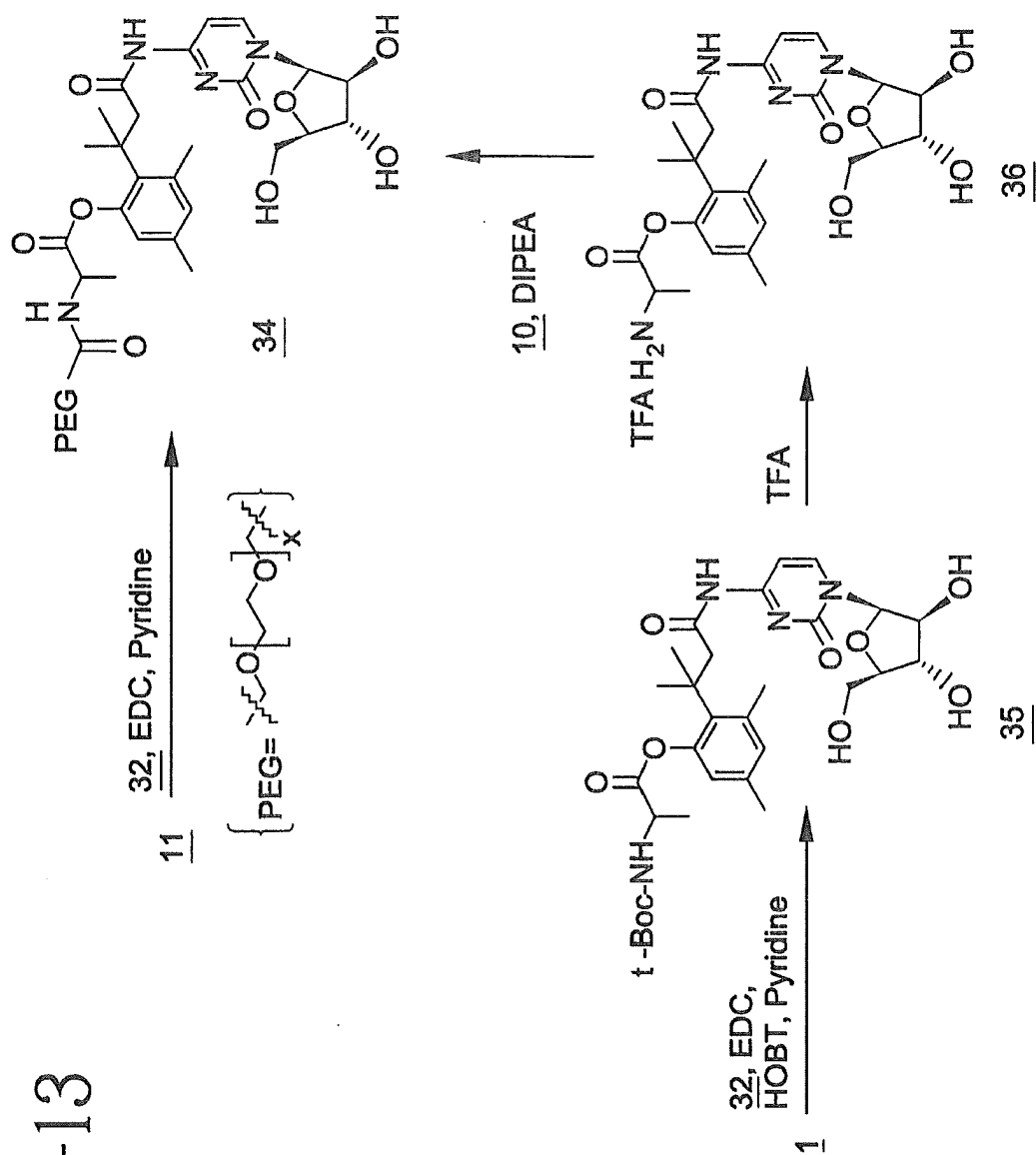
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FIG-12



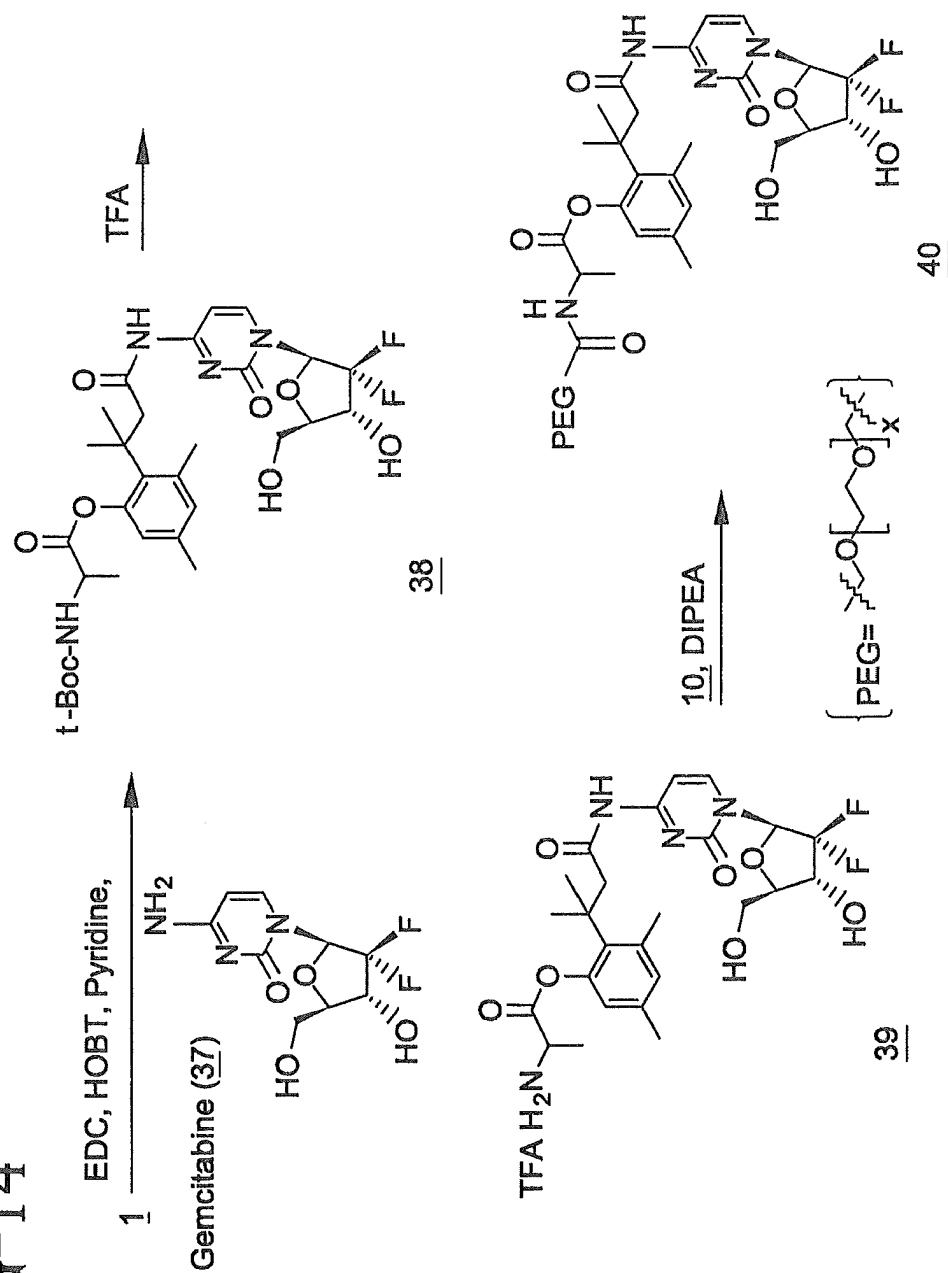
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FIG-13



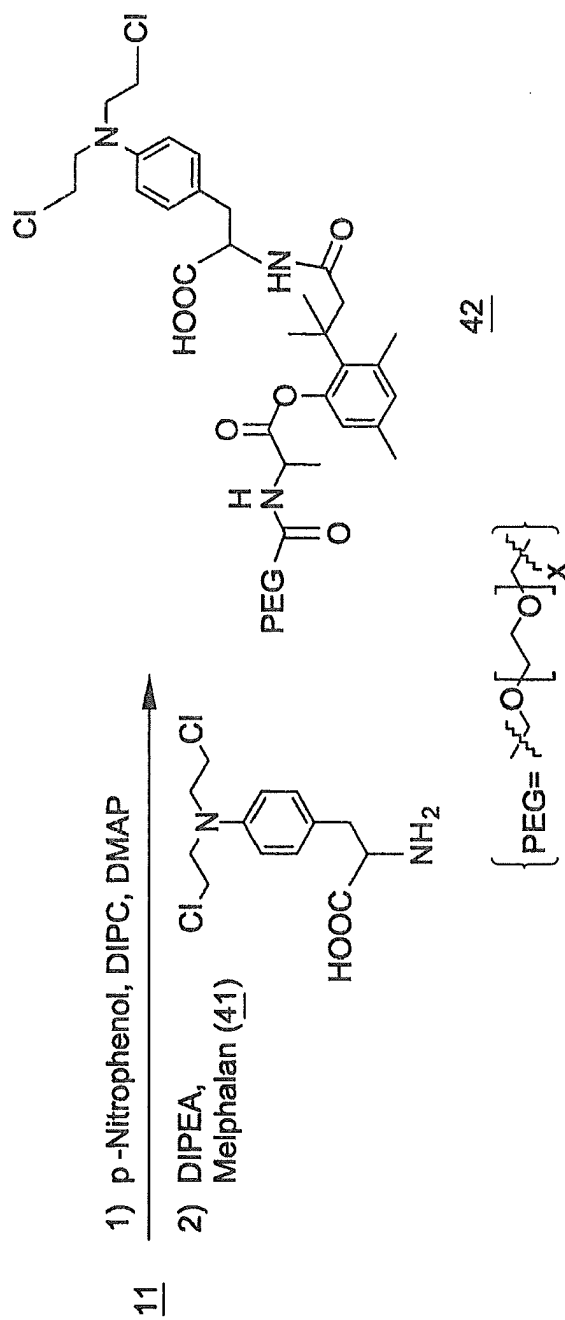
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FIG-14



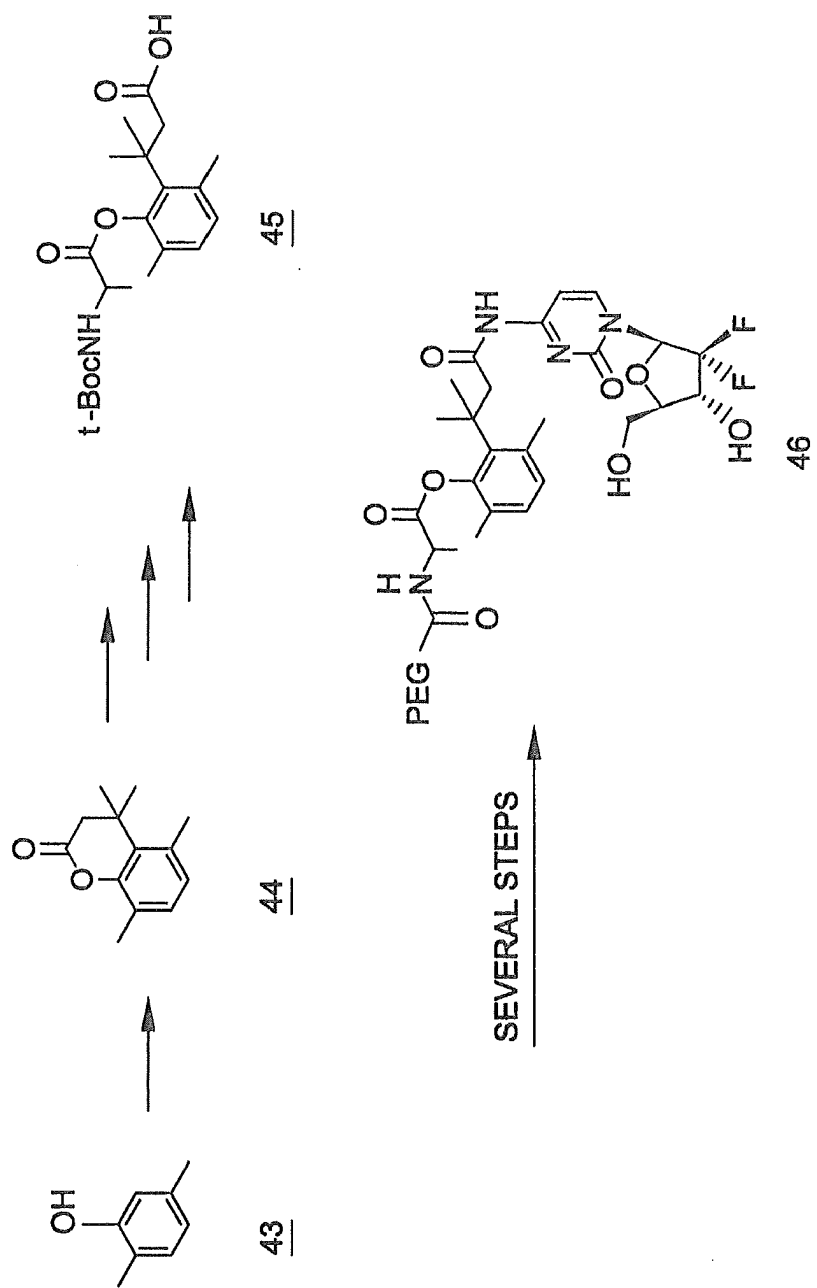
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FIG-15



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FIG-16



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/27805

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00

US CL :530/345, 402

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/345, 402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,672,584 A (BORCHARDT et al.) 30 September 1997.	1-34
A	SHAN et al. Prodrug Strategies Based on Intramolecular Cyclization Reactions J. Pharm. Sci. July 1997. Vol. 86, No. 7.	1-34



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 MARCH 1999

Date of mailing of the international search report

12 APR 1999

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